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BIOLOGICAL FUNCTIONS OF THE NOVEL LYSOPHOSPHATIDIC ACID (LPA) RECEPTOR, LPA4/p2y9/GPR23

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BIOLOGICAL FUNCTIONS OF THE NOVEL LYSOPHOSPHATIDIC ACID (LPA) RECEPTOR, LPA_4/p2y9/GPR23

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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List of Abbreviations

ATX	Autotaxin
cAMP	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase 2
DAG	1,2-diacylglycerol
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium
Edg	Endothelial differentiation gene
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine 5'-diphosphate
GFP	Green fluorescent protein
GPCR	G protein-coupled receptor
GROα	Growth regulated oncogene alpha
GST	Glutathione S transferase
GTP	Guanine 5' –triphosphate
IP_3	Inositol 1, 4, 5-triphosphate
IL-6, IL-8	Interleukin 6, Interleukin 8

KO	Knockout
LPA	Lysophosphatidic acid (18:1; 1-oleoyl-2-hydroxy-sn-glycero-3-phosphate)
LPA_1, LPA_2, LPA_3	Lysophosphatidic acid receptor 1, 2, 3
LPC	Lysophosphatidylcholine
LPP-1, LPP-2, LPP-3	Lipid phosphate phosphohydrolases 1, 2, 3
LysoPLD	Lysophospholipase D
MAG	Monoacylglycerol
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblasts
NPP	Nucleotide pyrophosphatase/phosphodiesterase
P2Y	Purinergic receptor family
PA	Phosphatidic acid
PAK-1	p21-activated kinase
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PCR	Polymerase chain reaction
PGE2	Prostaglandin E2
PI3K	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLA2	Phospholipase A2

PLC	Phospholipase C
PLD	Phospholipase D
PPARγ	Peroxisome proliferators-activated receptor γ
RNA	Ribonucleic acid
Rho-GAPs	Rho GTPase activating proteins
Rho-GEFs	Rho-specific guanine nucleotide exchange factors
S1P	Sphingosine-1-phosphate
SD	Standard deviation
SRF	Serum response factor
SRY	Sex-determining Region Y
uPA	Urokinase plasminogen activator
UTR	Untranslated region
VEGF	Vascular endothelial growth factor
WT	Wild type

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Abstract

BIOLOGICAL FUNCTIONS OF THE NOVEL LYSOPHOSPHATIDIC ACID (LPA) RECEPTOR LPA_4/p2y9/GPR23

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

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Lysophosphatidic acid (LPA), a naturally occurring phospholipid present in serum and malignant effusions, elicits diverse biological functions through binding and activating specific cell surface G-protein coupled receptors. In addition to the conventional LPA_1/Edg2, LPA_2/Edg4 and LPA_3/Edg7 receptors of the endothelial differentiation gene (Edg) family, LPA_4/p2y9/GPR23 of the purinergic receptor family and the related LPA_5/GPR92 and LPA_6/p2y5 have been identified as novel LPA receptors. These newly identified LPA receptors are structurally distant from the Edg LPA_{1-3} receptors and couple to G_q, $G_{12/13}$ and probably G_s subunits. However, the roles of the LPA_{4-6} receptors in LPA

signal transduction and physiology are poorly understood. This project has used biochemical and genetic approaches to study biological functions of LPA_4.

In the first part of the study, we confirmed that LPA_4 is indeed a functional LPA receptor mediating some cellular and biochemical responses to LPA including stimulation of neurite retraction, protein tyrosine phosphorylation. LPA_4 also physically binds to LPA when ectopically expressed in cell lines.

Mammalian cells usually express multiple LPA receptor subtypes and respond to LPA, making it difficult to link LPA receptors to specific responses. Targeted deletion has become a necessary approach to probe functions of individual LPA receptors. We therefore disrupted LPA_4-encoding gene (*lpa4/p2y9/gpr23*) in mice. LPA_4-deficient mice were born at the expected frequency and displayed no apparent abnormalities at least at early ages, indicating that LPA_4 is not required for fertility, embryonic development or normal physiology. This is similar to knockouts of other LPA receptors. The backup and/or redundant receptor subtypes of LPA may suffice to compensate for the loss of individual LPA receptors *in vivo*. Alternatively, LPA may not be the only or rate-limiting mediator physiologically required *in vivo*. LPA signaling may be more critical in pathophysiological conditions when levels of the lipid mediator are locally and temporally altered. The availability of LPA_4-null mice provides a valued model to analyze the roles of LPA_4 in pathophysiological processes.

Despite the lack of apparent phenotypes in mice, we took advantage of the LPA_4-negative mouse embryonic fibroblasts (MEFs) to evaluate the effects of *lpa4* deletion on cellular responses to LPA. Strikingly, LPA_4-deficient MEFs were hypersensitive to LPA-

induced migration. Consistent with negative modulation of the phosphatidylinositol 3 kinase (PI3K) pathway by LPA_4, LPA_4 deficiency potentiated AKT and Rac but decreased Rho activation induced by LPA. Reconstitution of LPA_4 converted LPA_4-negative cells into a less motile phenotype. In support of the biological relevance of these observations, ectopic expression of LPA_4 strongly inhibited migration and invasion of human cancer cells. When coexpressed with LPA_1 in B103 neuroblastoma cells devoid of endogenous LPA receptors, LPA_4 attenuated LPA_1-driven migration and invasion, indicating functional antagonism between the two subtypes of LPA receptors. These results provide genetic and biochemical evidence that LPA_4 is a suppressor of LPA-dependent cell migration and invasion. LPA4 may thus play a role in negative regulation of LPA signal transduction and specific cellular responses.

CHAPTER 1: GENERAL INTRODUCTION

Lysophosphatidic acid (LPA)

In the past decade, there have been considerable advances in our understanding of the sources and biological roles of lysophospholipids. The best characterized of these are LPA with a glycerol backbone and the related sphingosine-1-phosphate (S1P) containing sphingosine (Fig. 1-1). These lysophospholipids are not only metabolites in biosynthesis of more complex lipids in eukaryotic and prokaryotic cells but also have emerged as pluropotent intercellular mediators to induce hormone- and growth factor-like responses in their target cells through activation of specific G protein-coupled receptors.

LPA is the simplest lysophospholipid made up of a glycerol backbone with a phosphate group, and a long saturated or unsaturated fatty acyl chain (Fig.1-1). It is unique from the other lysophospholipids because it lacks a head group attached to its phosphate moiety, such as the choline head group in lysophosphatidylcholine. Despite its simple structure, LPA, which was originally considered as a precursor and component of lipid remodeling, is now widely known to be an important extracellular mediator of a broad variety of biological processes by binding to its cognate G protein-coupled receptors (GPCRs) (1). The most prominent cellular effects of LPA include stimulation of cell proliferation, survival (2), migration (3,4), cytoskeleton remodeling (5,6) in both normal and neoplastice cells. LPA is also involved in control of adipogenesis and obesity by stimulating proliferation of pre-adipocytes through LPA_1 thereby inhibiting its maturation

to fat cells (7-9). As a bioactive constituent of mildly oxidized LPL and atherosclerotic lesions (10), LPA has been postulated to play a role in the pathogenesis of atherosclerosis particularly through its activity on other vascular effect such as stimulation of monocyte attachment to blood vessel walls (11), plaque formation (12), increased endothelial permeability (13,14) and promotion of vascular smooth muscle cells dedifferentiation (15). Most importantly, LPA induces progressive formation of neointima in a rat carotid artery model (16,17). These findings suggest that LPA may act as an endogenous atherogenic factor *in vivo*. Recent studies demonstrate that LPA modulates expression of many paracrine growth factors including those involved in inflammation, angiogenesis and tumor progression such as interleukin 6 (IL-6), interleukin 8 (IL-8), growth regulated oncogene α (GROα), cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF), urokinase plasminogen activator (uPA) (18-24). LPA may thus promote tumorigenesis by upregulating its target genes to create a more invasive and metastatic microenvironment for tumor cells.

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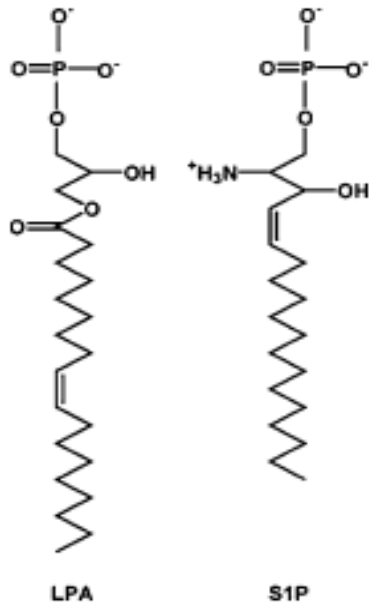


Figure 1-1. Chemical structures of the bioactive lysophosphatidic acid (LPA) and its related sphingosine-1-phosphate (S1P).

LPA Biosynthesis and Metabolism

LPA is produced and secreted by activated platelets in response to either coagulation or wound healing, thus LPA is a active constituent of serum and present at physiologically relevant concentrations in an albumin-bound form (25-29). The physiological concentrations of LPA in serum range from approximately 1 to 10 μM. LPA is also rich in body fluids such as saliva, follicular fluids and malignant effusions (30-33). In ascites of ovarian cancer patients, the levels of LPA reach 80 μM. Ovarian tumor cells have the ability to synthesize and secrete LPA, which may contribute to the high levels of LPA accumulated in ascites of ovarian cancer patients (33,34). LPA can also be produced by other cell types such as adipocytes, fibroblasts, endothelial cells, and leukocytes (35). Extracellular lipoprotein oxidation of LDL can also lead to production of LPA (10).

The molecular pathways for extracellular LPA production have been best characterized in activated platelets that are responsible for increased LPA levels in serum compared to the whole blood or plasma. LPA can be produced by activated platelets from newly generated, membrane associated PA by the action of phospholipase D followed by phospholipase A1 or A2 -mediated deacylation (28). However, the bulk of LPA arising from platelet activation results from the sequential cleavage of serum and membrane phospholipids to lysophospholipids by PLA1 and PLA2 secreted by platelets (27,28), followed by conversion to LPA by lysophospholipase D (LysoPLD) present in the plasma (11,27). It has been long known that plasma contains prominent LysoPLD activity, contributing to ongoing production of LPA from lysophosphatidylcholine (LPC) during cell culture (27,29). The plasma LysoPLD was recently identified to be autotaxin (ATX)

(3,11), a member of the nucleotide pyrophosphatase and phosphodiesterase family of exo- and ecto-enzymes (36). Homozygous deletion of ATX leads to embryonic lethality at E9.5 due to impaired vessel formation in the yolk sac and embryo proper (37,38), indicating that ATX is essential for embryonic vasculature. The results also imply that ATX is necessary for LPA biosynthesis at least during embryo development. ATX, a 125 kDa glycoprotein was first isolated in 1992 as an autocrine motility-stimulating factor for human melanoma cells (39). Now, it has become clear that ATX promotes cell migration and invasion via formation and action of LPA (40,41).

LPC, the major physiological substrate of ATX, is secreted by the liver and circulate at high concentration level (>100 μM) in the blood in an albumin-bound form (35,42). The precursor of LPC, PC is also abundant in the plasma reaching approximately 1 mM (35). Despite the high concentrations of LPA precursors in the blood stream, LPA concentrations in the plasma are maintained at low levels under physiological conditions. This could be explained by the fact that ATX catalyzing activity is inhibited by multiple factors including its product LPA (negative feedback), certain amino acids and other blood constituents (43).

LPA can be metabolized through acylation of the sn-2 hydroxyl group by LPA acyl transferases such as endophilin (44), deacylated by lysophospholipases to produce glycerol phosphate (45), or dephosphorylated by lipid phosphate phosphohydrolases (LPP) including LPP1/PAP2A, LPP3/PAP2B and LPP2/PAP2C to generate monoacylglycerol (46-49) (Fig. 1-2). LPPs are located at the cell membrane with the catalytic domain facing the extracellular media. The enzymes degrade LPA associated with the external leaflet of

the membrane (50). Overexpression of LPPs has been shown to antagonize cellular responses to LPA and reduce LPA concentrations in the medium (50). In contrast, inhibition of LPP enzyme activity sensitizes platelets to LPA-induced responses as well as increases thrombin-induced LPA production (51). Overall, these observations suggests LPPs play a critical role in controlling extracellular levels of LPA or membrane accessible LPA.

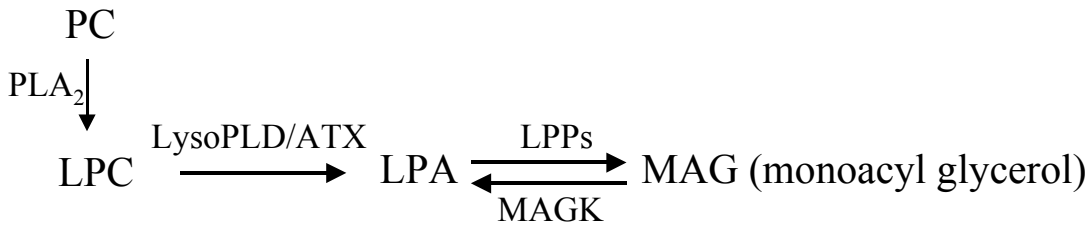


Figure 1-2. Regulation of LPA levels in the extracellular environment by ATX and LPPs. Secreted ATX/lysoPLD hydrolyzes carrier-bound and membrane-associated LPC to generate LPA. Newly produced LPA acts on its G protein–coupled receptors and thereby evokes numerous cellular responses. Excess LPA is converted into monoacylglycerol (MAG) by membrane-bound lipid phosphatases (LPPs).

The LPA Receptors

The biological actions of LPA are mediated by a number of G protein-coupled receptor(s) (GPCRs) (1). To date, six cell surface GPCRs named LPA_{1-6} have been identified as the receptors for LPA (1,5,52-56). Based on the amino acid homology (57), the LPA receptor family can be divided into two subfamilies: one is composed of the first three LPA receptors: LPA_{1-3} of the Edg family, and the other consists of LPA_4/p2y9/GPR23, LPA_5/GPR92 and LPA_6/p2y5. LPA_{1-3} share about 50-57% amino acid sequences similarity, with their C-terminal tails being most divergent (5,52,53). The other GPCRs of the Edg family are receptors for the related S1P (58). Three novel LPA receptors, LPA_{4-6} are structurally distant from the canonical Edg LPA receptors (54,55,59,60). LPA_4 and LPA_6 are members of the purinergic receptor family. They are closely related to the nucleotide receptors P2Y1, P2Y4 and P2Y6 (61). LPA_5 belongs to the purinocluster of GPCRs which consist of the formyl peptide receptors (FPRs), receptors for nucleotides (P2Ys), thrombin, and leukotriene and a large number of orphan receptors. LPA_{4-6} display around 31% homology whereas only shares 21.3% - 22.6% homology with the Edg LPA receptors (54-56). Since the sequence identity within this purin receptor branch is generally low (~20%), LPA_{4-6} considered to be highly related. Thus LPA receptors are derived from two different ancestor genes.

The first cDNA encoding a functional LPA receptor, now referred exclusively as LPA_1/Edg2, was cloned from mouse cortical neuroblast cell lines in 1996 (5). It was named *ventricular zone gene-1* (*vzg-1*) because of its predominant expression in the neurogenic ventricular zone of the embryonic cortex. Among the LPA receptors, LPA_1 is

the best characterized subtype and most widely expressed in almost all mammalian tissues with the exception of livers (1,62). In some cell lineages, LPA_1 is the major regulator of cell motility by triggering Rac and Rho dependent cytoskeleton changes. LPA_1-deficient mice showed partial postnatal lethal (~50%) partly due to a suckling defect resulting from impaired olfaction (63). However, these animals proved to be valued models to study LPA signaling in pathophysiological conditions. This has led to identification of novel roles for LPA_1 in the initiation of neuropathic pain and formation of pulmonary and renal fibrosis (64-66). In addition, LPA signaling in $LPA_1^{-/-}$ MEFs was significantly compromised or abolished (62).

LPA_2/Edg4 and LPA_3/Edg7 were identified as LPA receptors following the discovery of the LPA_1 receptor. (52,53). Gene structure analysis indicates that all three Edg LPA receptors contain the conserved introns in transmembrane domain six (67-69). Despite high homology among them, LPA_{1-3} have distinct tissue distribution and functions. For example, LPA_2 shows more restricted expression in adult tissues compared to LPA_1 (70). LPA_2 is frequently overexpressed in human cancers such as ovary, breast, colon, pancreas and thyroid cancers (19,33,71-77). It should be noted that the first reported human *lpa*$_2$ clone was derived from an ovarian tumor library (52) and contained a frame-shift mutation that produced 31 extra amino acids at its intracellular carboxyl terminal end, which could produce a gain of function mutant (78). Several 3'-untranslated region (UTR) variants of the *lpa*$_2$ transcripts have been found in some tumors, suggesting oncogenic potential may be conferred by altered LPA_2 stability/signaling (69). Targeted deletion of *lpa*$_2$ in mice does not have obvious phenotypes (79). However, significant attenuation of

LPA signaling (e.g. PLC activation, Ca^{2+} mobilization, and stress fiber formation) was observed in primary cultures of MEFs (79). The *lpa*$_1$ *lpa*$_2$ double-null mice do not show additional phenotypic abnormalities beyond those attributable to *lpa*$_1^{(-/-)}$ except for an increased incidence of perinatal frontal hematoma (79). However many LPA-induced responses including cell proliferation, adenyl cyclase activation, PLC activation, Ca^{2+} mobilization, JNK and AKT activation, and stress fiber formation, were absent or severely reduced in double-null MEFs (79). All these responses were only partially affected in MEFs lacking either LPA_1 or LPA_2 (79). These results indicate that LPA_2 may act redundantly with LPA_1 to mediate most LPA responses.

Expression of LPA_3 is hardly detectable in most adult tissues (70). However, similar to LPA_2, LPA_3 expression is elevated in malignancies including prostate, ovarian and pancreatic cancers (77,80-82). Targeted deletion of *lpa*$_3$ in mice results in delayed implantation and altered embryo spacing (83). This could be caused by the downregulation of COX-2 in uterine. Implantation defect could be partially rescued by exogenous administration of PGE2 into *lpa*$_3$ deficient female mice. The phenotype of *lpa*$_3$ KO mice is similar to that of $cPLA_{2\alpha}$ knockout mice, suggesting a critical role of LPA_3 in regulation of COX-2 expression and PGE2 production in the female reproductive system.

The $LPA_{4\text{-}6}$ receptors are poorly characterized compared to the Edg LPA receptors. (54-56). None of these receptors have been knockout in mice. LPA_4 is expressed at modest levels in majority of the tissues including heart, skeleton muscle, testis, ovary and embryonic stem cells (59). LPA_5 is highly expressed in small intestines, sensory dorsal

root ganglia as well as embryonic stem cells (55). LPA_6 is required for maintenance of human hair growth and mutated in hypotrichosis simplex (56).

In addition to these cell surface GPCRs, LPA has been shown to bind and activate the peroxisome proliferator–activated receptor γ (PPARγ) which plays critical roles in controlling fat and energy metabolism (84,85).

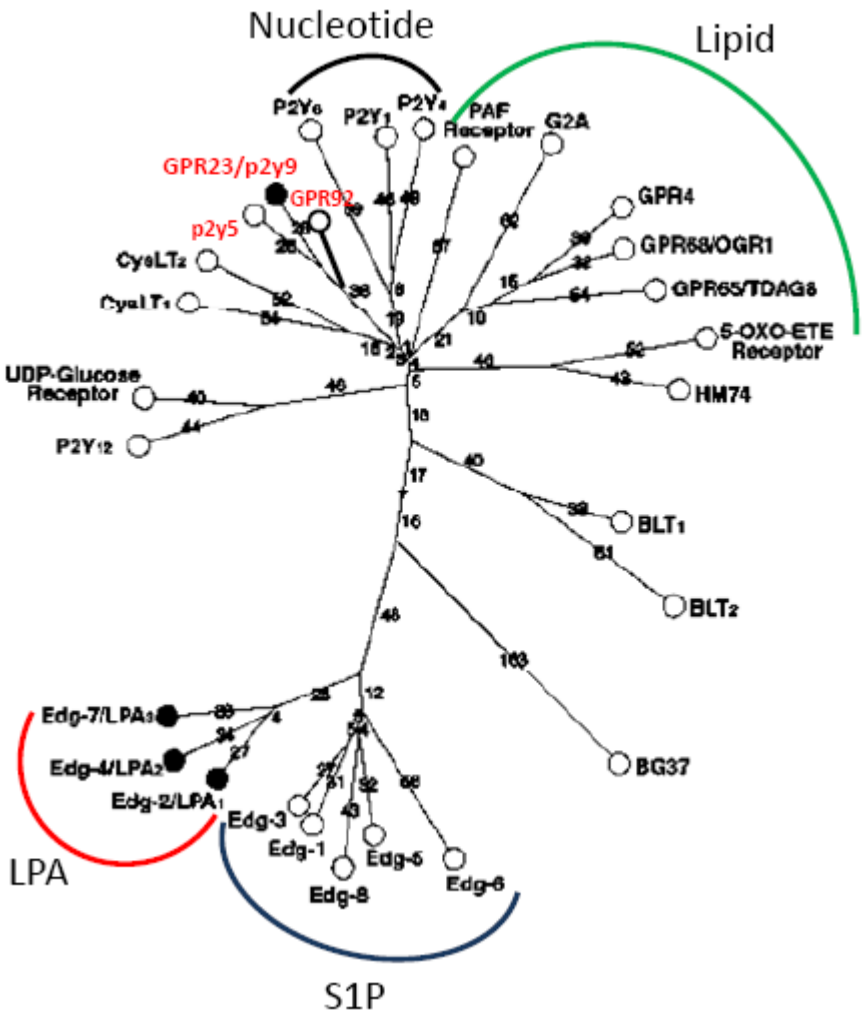


Figure 1-3. Phylogenetic tree of selected human GPCR showing protein sequence relationship of the human LPA and S1P receptors. The values indicate the lengths of the branches. The sequence divergence between any pair of sequences is equal to the sum of the value of the branch lengths. A small dot indicates the centroid of the tree. Shorter lengths mean more closely related genes.

Signal Transduction of LPA Receptors

The heterotrimeric G proteins serve as important molecular switches that connect extracellular signals from GPCRs on the cell membrane to downstream effectors. There are four major classes of G protein alpha subunits that are coupled to LPA receptors: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$. The major signaling routes downstream of G proteins are summarized in Fig. 1-4. These include (1) G_i-mediated activation of Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) - AKT/Rac cascades. Activation of Rac and AKT/PKB promotes forward cell movement and cell survival, respectively; (2) $G\alpha_q$-mediated activation of phospholipase C (PLC) and the subsequent hydrolysis of phosphatidylinositol-(4,5)-bisphosphate (PIP_2) and Ca^{2+} mobilization from ER. (3) $G\alpha_{12/13}$-mediated activation of the small GTPase Rho, which promotes contraction of the actomyosin-based cytoskeleton leading to changes in cell shape. (4) $G\alpha_s$-mediated adenylate cyclase activation with a subsequent increase in cAMP levels leading to activation of cAMP dependent protein kinase A (PKA). It has been shown that LPA_1 and LPA_2 interact with G_i, G_q and $G_{12/13}$ while LPA_3 only interacts with G_i and G_q (1). $LPA_{4\text{-}5}$ appears to couple to G_s, in addition to G_q and $G_{12/13}$ (54,55,59,60). By acting on its specific GPCR, LPA can exert diverse biological effects including stimulation of cell growth and survival, cytoskeleton remodeling, and promotion of cell motility.

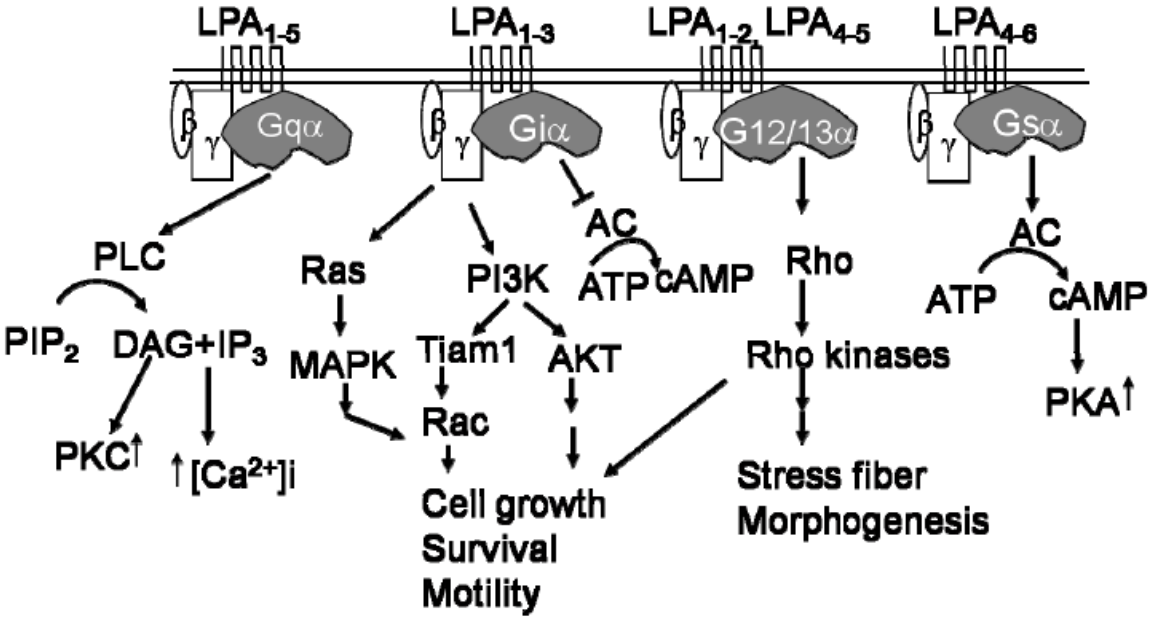


Figure 1-4. LPA receptors and signal transduction. LPA binding to its cognate receptors leads to activation of specific intracellular G proteins and the subsequent activation of multiple signaling cascades. The diagram outlines the signaling pathways that are linked to the well-characterized biological functions of LPA such as stimulation of cell proliferation, survival and motility.

LPA and Cell Motility

Stimulation of cell migration and invasion is one of the major biological functions of LPA and its producing enzyme ATX. ATX was originally identified as a tumor cell motility-stimulating factor (39). LPA- and ATX-induced cell migration is mediated mainly by the LPA_1 receptor although LPA_2 or LPA_3 may be also capable of evoking the response in various cellular contexts.

LPA is involved in regulation of the small Rho GTPases, RhoA, Cdc42 and Rac, all of which are crucial modulators of cell motility (86). Specifically, RhoA mediates actomyosin-driven contractility and stress fibers formation and Cdc42 controls filopodia formation, whereas Rac regulates lamellipodia protrusion and drives forward cell movement. LPA activates RhoA via $G\alpha_{12/13}$ subunits (87) which bind directly to Rho-specific guanine nucleotide exchange factors (Rho-GEFs) including p115-RhoGEF, LARG and PDZ-Rho-GEF. These exchange factors promote RhoA-GTP accumulation and initiate activation of Rho-associated coiled-coil containing protein kinase 1 (ROCK). ROCK promotes actin polymerization and inhibits myosin light chain phosphatases (PP1M), thereby increasing phosphorylation of myosin light chain (MLC) by MLC kinase and actomyosin contraction (88). The actomyosin-based cell contraction underlies neurite retraction, cell rounding, and endothelial tight junction opening (13,89,90). On the other hand, following initial cell rounding, LPA promotes cell spreading, lamilipodia formation and cell movement by activating the Rac GTPase through an LPA_1-G_i-mediated pathway that involves PI3K activity and the guanine nucleotide exchange factor Tiam1 (91).

Cell migration propelled by repeated cycles of protrusion at the leading edge and retraction at the trailing end has to be coordinated with cell adhesion to allow directional movement. In this respect, although Rac and Rho are signaled through distinct G protein pathways, they affect each other's activity (92). In fibroblasts, activation of Rac has been shown to specifically down-regulate Rho activity (93). In keeping with this, fibroblasts from Tiam1-deficient mice shows enhanced RhoA activity, stress fiber formation and cell rounding in response to LPA (91). This may be explained by Rac-mediated production of reactive oxygen species (ROS) leading to activation of p190RhoA-GAP hence reduction of RhoA-GTP levels (94). Likewise, excessive Rho also inhibits Rac activation (95). Therefore, cell migration is coordinated by relative activities of Rac and Rho. Little is known about how the migratory response to LPA is appropriately controlled in mammalian cells that usually coexpress multiple LPA receptor subtypes of different abilities to activate Rac and Rho.

CHAPTER 2: LPA_4/p2y9/GPR23 AS A NOVEL RECEPTOR OF LPA

Introduction

LPA had been a subject of extensive research in signal transduction and physiology (2,6,96-99). Although there was strong evidence for the involvement of specific GPCRs in biological actions of LPA (100), the search for LPA receptors had been unsuccessful until 1996 due to lack of specific receptor antagonists, difficulty in ligand-binding experiments and the ubiquitous presence of LPA response in most cell types (101). The first LPA receptor, LPA_1 was cloned and identified from mouse cortical neuroblasts cDNA (5). Following the discovery of the first LPA receptor, the other two Edg LPA receptors (LPA_2 and LPA_3) were identified based on the sequence similarities to LPA_1. Other five members of the Edg family are found to be specific receptors for S1P. The Edg LPA receptors mediate many physiological functions of LPA. However, there is evidence that additional non-Edg LPA receptors might exist. For examples, in embryonic fibroblasts from LPA_1 and LPA_2 double knockout mice, certain responses to LPA such as inositol phosphate production, adenylyl cyclase inhibition and stress fiber formation were reduced but not eliminated. (79). In these cells, LPA_3 mRNA was not expressed, suggesting the presence of extra LPA receptor(s). In addition, skin fibroblasts from LPA_1 and LPA_2 double knockouts, LPA remained capable of stimulating partial activation of Rho (62).

Further evidence stems from targeted deletions of LPA_{1-3} receptors. Only minor phenotypic changes were seen in LPA_1 or LPA_2 receptor-deficient mice (63,79). Homozygous deletion of the LPA_3 receptor leads to a delayed implantation and defective embryo spacing, associated with reduced uterine expression of COX-2 mRNA (83). While these roles for individual receptors have been identified, more profound effects such as early embryonic lethality have not been observed from single or even double receptor knockouts. These results are in contrast to deletion studies of the LPA synthesizing enzyme autotoxin (ATX) where homozygous deletion results in embryonic lethality at E9.5 due to impaired vessel formation in the yolk sac and embryo proper (37,38). These results suggest involvement of other yet unidentified receptors as effectors of ATX.

In 2003, the fourth LPA receptor LPA_4/p2y9/GPR23 of the purinergic receptor (P2Y) family was described by Dr. Shimizu's group (54). This putative LPA receptor was notable for its low amino acid sequence homology with the three well studied Edg LPA receptors, and its ability to increase cAMP production by coupling to $G\alpha_s$ subunit (54). Furthermore, LPA_4 was reported to be highly expressed in the ovary (54).

Because of the structural difference from the Edg LPA_{1-3} receptors, there has been skepticism about whether LPA_4 is a bona fide LPA receptor. Misidentification of receptors for LPA and other bioactive lipids has occurred previously (102-106). Therefore, independent confirmation of LPA_4 identity as a functional LPA receptor is necessary before further analysis of its role in LPA signal transduction. In this section of my study, I performed a number of assays to establish that LPA_4 is indeed a functional LPA receptor.

Different from the Edg LPA receptors, however, LPA_4 mediates only a subset cellular response to LPA.

Materials and Methods

Reagents

1-Oleoly (18:1) LPA and [^{3}H]-1-Oleoly (18:1) LPA was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Prior to use, LPA was dissolved in PBS containing 0.5% fatty acid-free bovine serum albumin (BSA). BSA and protease inhibitor cocktail tablets were purchased from Roche (Indianapolis, IN). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). All oligonucleotides and primers were synthesized by Operon Biotechnologies, Inc (Huntsville, AL). TRIzol and cell culture medium were obtained from Invitrogen Inc. (Carlsbad, CA). Fetal bovine serum (FBS) was from Biomeda (Foster City, CA). Anti-pan tyrosin phosphorylation, anti-tubulin, anti-Paxillin, and anti- GSK3α/β antibody were obtained from Cell Signaling (Danvers, MA). Polybrene and anti β-actin monoclonal antibody were obtained from Sigma (St. Louis, MO). The rabbit polyclonal antibody against the C-terminus of the human LPA_4 was kindly provided by Dr. T Shimizu (University of Tokyo). Luciferase assay reagents were obtained from Promega (Madison, WI).

Cell culture

The B103 rat neuroblastoma cell line lacking endogenous LPA receptors was obtained from Dr. J. Chun (Scripps Research Institute) and cultured in DMEM (Invitrogen)

supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (107). NIH 3T3, Rat hepatoma 7777, and the ecotropic envelope-expression packaging cell line Bosc23 cells were obtained from ATCC (Manassas, VA) and cultured as recommended by ATCC. All the cell lines were frozen at early passages and used for less than 10 weeks in continuous culture.

Construction of Retrovirus Expression Vectors

The human LPA_4 and LPA_1 cDNAs were inserted between *BamH I* and *Xho I* sites upstream of the internal ribosomal entry site of the Moloney murine leukemia retrovirus vector LZRS-EGFP (a gift of J. Chun, Scripps Research Institute) (107). The structure and the insert sequences were confirmed by restriction digestion and automatic sequencing.

Generation of the LPA_4 and LPA_1 retrovirus and infection of target cells

The Bosc23 packaging cells were transfected with LZRS-EGFP, LZRS-EGFP-LPA_4 or LZRS-EGFP-LPA_1 using Lipofectamine 2000 following the protocol of the manufacturer (Invitrogen). Approximately 20 hours after the beginning of transfection, the cells were fed fresh DMEM supplemented with 10% FBS. Culture supernatants containing retrovirus were harvested 48 hours late, cleared by brief spin and used to recipient or stored at –80°C.

The recipient cells in 35-mm dishes at around 50% confluence were incubated for 16 hours with 1.5 ml of viral supernatants containing 8 μg/ml of Polybrene (Sigma). The

positive cells were isolated by FACS and expanded as stable lines expressing LPA_4 or LPA_1.

LPA binding assay

NIH 3T3 or Rat hepatoma 7777 cells stably transduced with LPA_4, LPA_1 or control retrovirus were starved overnight, and then harvested in binding buffer 25 mM HEPES [pH 7.5] containing 20 μM A-PMSF and protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were then sonicated three strokes with 20 sec on and 1 min off at level 2. The sonicated samples were first cleared at 1000 × g for 10 min at 4 °C and the supernatant was further centrifuged at 10,000 × g for 1 hr at 4 °C. The pallets were resuspended with binding buffer and homogenized using a Dounce homogenizer.

Membrane samples (40 μg) was incubated with 1 μM [^{3}H]-LPA (1-oleoyl-[9, 10-^{3}H]-LPA, 47 Ci/mmol,) in LPA-binding buffer containing 0.1 % fatty acid-free BSA (Sigma) for 1 hr on ice. The bound [^{3}H]-LPA was collected onto a GF/B glass fiber filter (Whatman, NJ). The filter was then washed four times with PBS on 1225 sampling manifold (Millipore, MA) and dried for 1 hr at 65 °C. Radioactivity on the membrane was measured in a liquid scintillation counter. To examine the specificity of LPA binding, the amounts of LPA bound (using 1 μM of [^{3}H]-LPA) in the presence of excess nonradioactive LPA (10 μM) were determined. Total and nonspecific binding was evaluated in the absence and presence of 10 μM cold LPA, respectively. The specific binding was calculated by subtracting the nonspecific binding value (cpm) from the total reading (cpm).

Western Blotting

Cells were lysed in SDS sample buffer (2% SDS, 62.5 mM Tris [pH 6.8], 10% (w/v) glycerol and 5% (w/v) 2-mercaptoethanol). Total cellular proteins were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane (Immobilon-P), and immunoblotted with antibodies following the protocols of the manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Amersham Biosciences) using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling).

Neurite retraction

B103 cells were seeded in poly-D-lysine-coated 6-well plates (1.5×10^5/well). After 24 hr incubation to allow attachment to the plates, the cells were shifted to serum-free DMEM for 16 hr. The cells were then treated with 1 μM LPA for 15 min and photographed using Zeiss microscope connected to a Canon digital camera. Cells with spherical shape lacking neurite longer than cell body and were scored as rounded cells. At least three different fields per well were examined with a minimum of 200 cells scored. The LPA-induced neurite retraction was expressed as percentages of round cells versus the total number of the cells.

Chemotaxis

Chemotactic migration of cells was measured with transwell chambers made of polyethylene terephthalate (BD bioscience, 8 μM pore size). The transwells were coated

with collagen I. LPA was added to the lower chamber, and cells were loaded to the upper chamber at 1×10^4 cells/well. After 4 hr, nonmigrated cells on the upper chambers were removed by cotton swabs and the cells that traversed and spread on the lower surface of the transwell were washed in PBS and stained with 0.1% crystal violet. The migrated cells were counted with a microscope and a 20 × objective. Each data point is the average number of cells in five random fields, and the number is the average ± SD of triplicates, representative of 3 independent experiments.

Statistical analysis of data

Each data point was calculated from triplicate samples unless otherwise indicated. The data presented are mean ± SD. The statistical differences were determined by variance or Student's *t* test where $p<0.05$ was considered to be significant.

Results

Expression of LPA_4 mRNA increases with cell density

LPA_4 was reported to be highly expressed in the ovary (54). LPA signaling and LPA receptors have been implicated in the development of human malignancies, especially ovarian cancers, suggesting the highly expressed LPA_4 in the ovary may play a role in ovarian carcinogenesis. Therefore we first examined the expression of LPA_4 in ovarian cancer cell lines and normal ovarian epithelial cells. Consistent with previous observations (81), LPA_2 and LPA_3 are upregulated in ovarian cancer cell lines compared to normal ovarian epithelial cells (Fig. 2-1). LPA_1 expression was variable showing no consistent

changes between normal and cancer cells In contrast to the Edg LPA receptors, LPA_4 is absent or minimally expressed in ovarian cancer cell lines (Fig. 2-1).

We examined LPA_4 mRNA expression in other cell lines and found that it was expressed at significant levels in murine and human fibroblast (Fig. 2-2). Furthermore, LPA_4 expression in fibroblast was not static. Fig. 2-2 shows Northern blot analysis of LPA_4 mRNA levels in NIH 3T3 cells cultured at approximately 50, 75 and 100% confluence. Non-confluent NIH 3T3 cells expressed a relatively low level of LPA_4 mRNA. The abundance of the transcripts increased when the culture reached higher densities, suggesting that LPA_4 expression is not constitutive but rather regulated by cell conditions.

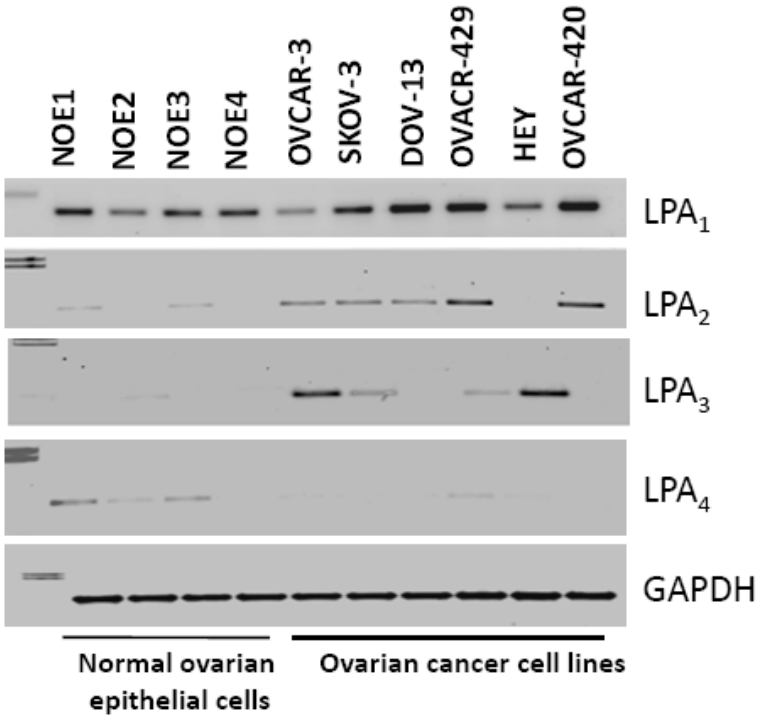


Figure 2-1. Expression of LPA_{1-4} receptors in normal ovarian epithelial cells and ovarian cancer cell lines. Expression of LPA_{1-4} receptors in normal ovarian epithelial cells (NOE) and six ovarian cancer cell lines was analyzed by RT-PCR. GAPDH was included as loading controls.

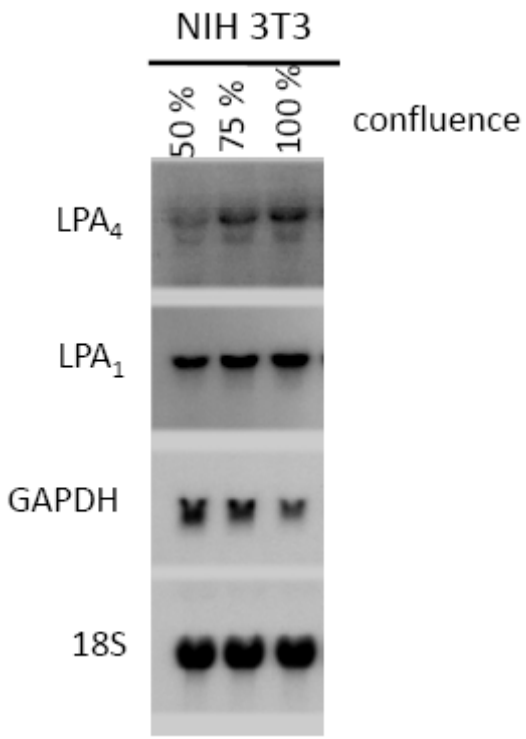


Figure. 2-2. Upregulation of LPA_4 mRNA by cell density in NIH 3T3 cells. Total cellular RNA was prepared from NIH 3T3 cells of different densities (50, 75 and 100% confluence). LPA_4 and LPA_1 mRNA levels were examined by Northern blotting analysis with ^{32}P-labeled LPA_4 or LPA_1 cDNA as probes. The blot was reprobed with GAPDH and 18S to confirm equal loading among samples.

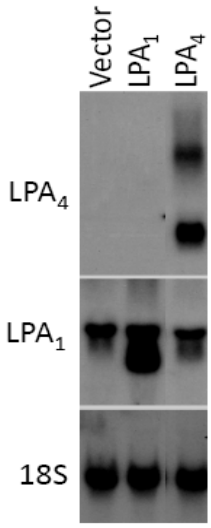
LPA binds to LPA_4

To determine whether LPA_4 indeed physically binds to LPA, we performed LPA binding assay with membrane fractions isolated from NIH 3T3 cells overexpressing LPA_4 or LPA_1. To overexpress LPA_4 or LPA_1 in NIH 3T3 cells, high titers of retrovirus produced in the Bosc23 packaging cell line was used to infect NIH 3T3 cells. The stably-transduced cells were isolated by fluorescence-activated cell sorting (FACS). Northern blotting analysis confirmed mRNA expression of LPA_4 or LPA_1 in the transduced cells (Fig. 2-3B)

Membrane fractions were prepared from control NIH 3T3 and LPA_1- or LPA_4-transduced NIH 3T3 cells and incubated with 1μM [^{3}H]-LPA in the presence or absence of 10 μM cold LPA. The specific binding between LPA and the membrane preparations was determined by extensive washing. Both LPA_1 and LPA_4-transduced membranes showed significantly increased binding to [^{3}H]-LPA compared to membranes isolated from control cells (Fig. 2-3B). To further confirm the physical binding of LPA_4 to LPA, we performed the binding assay, in Rh7777 which lack endogenous LPA receptors. LPA_1 or LPA_4 was transduced into Rh7777 cells via retrovirus-mediated transduction. When cultured in complete medium, the control Rh7777 cells showed a rounded, refractile appearance. In contrast, Rh7777-LPA_1 cells displayed a more flattened morphology. The cells expressing LPA_4 show morphological appearances between control Rh7777 and LPA_1-expressing cells, suggesting the exogenous expressed LPA_1 and LPA_4 are functional in Rh7777 cells (Fig. 2-4). As expected, control Rh7777 cells lacking endogenous LPA receptors showed a low background binding to [^{3}H]-LPA. Expression of either LPA_1 or LPA_4 greatly enhanced

[^{3}H]-LPA binding to membranes of retrovirus transduced Rh7777 cells (Fig. 2-5). These observations indicated that similar to the well characterized LPA_1 receptor, LPA_4 was indeed capable of binding to LPA.

(***A***)



(***B***)

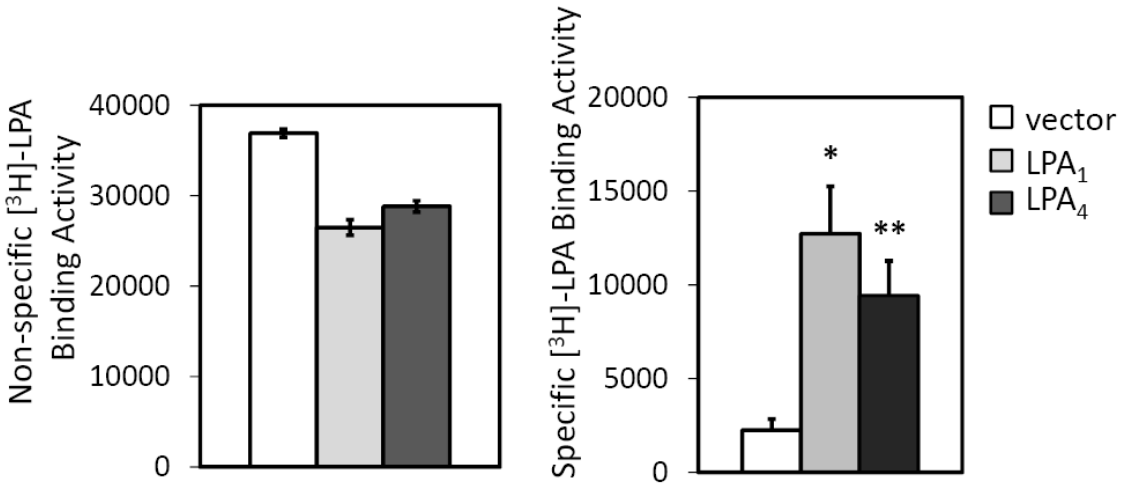


Figure 2-3. [^{3}H]-LPA binding to plasma membranes of NIH 3T3 cells expressing LPA$_4$. (***A***) RNAs (25 μg) from NIH 3T3 stably infected with control, LPA$_1$ or LPA$_4$ retrovirus were subjected to Northern blot analysis. 18S RNA served as loading control. (***B***) [^{3}H]-LPA non-specific and specific binding in plasma membranes of NIH 3T3 cells

expressing control vector, LPA_1 or LPA_4. Data are the mean ± SD (n =3). *, p = 0.0056; **, p = 0.0062 (Student's t test) *versus* empty vector expressing cells.

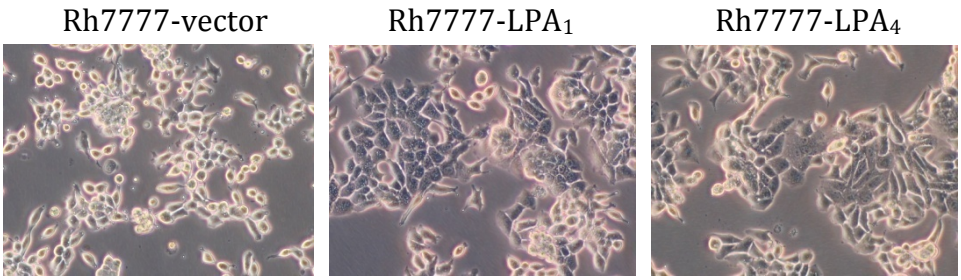


Figure 2-4. Microscopic morphology of control, LPA_1 or LPA_4-transduced Rh7777 cells. Phase-contrast microscopic photographs of Rat hepatoma 7777 cells stably transduced with vector control, LPA_1, or LPA_4 retrovirus.

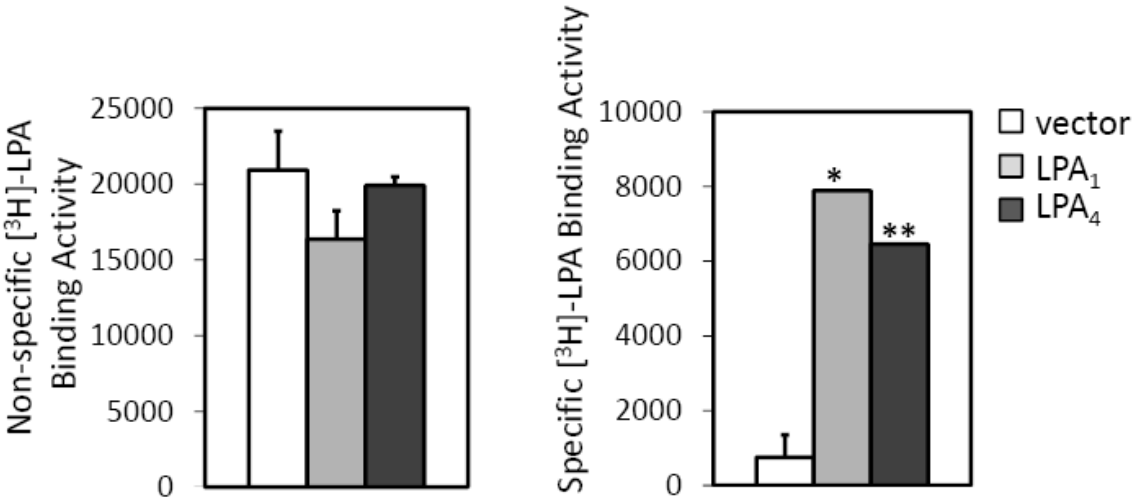


Figure 2-5. Physical binding of [^{3}H]-LPA to LPA$_1$ or LPA$_4$ ectopically expressed in Rh7777 cells. Membrane fractions (40 μg) from vector-, LPA$_1$- or LPA$_4$-transduced Rh7777 cells were incubated with [^{3}H]-LPA for 1 hour. Specific binding was assessed in the presence of 10 μM LPA. Data are presented as means ± SD (n =3). Asterisks indicate significant differences from the control. *, p = 0.0045; **, p = 0.0031 (Student's t test) *versus* empty vector expressing cells.

LPA_4 mediates LPA-induced protein tyrosine phosphorylation in Rh7777 cells

LPA stimulates protein tyrosine phosphorylation in fibroblasts and epithelial cells (108-111). To determine whether LPA_4 was capable of mediating tyrosine phosphorylation of intracellular proteins, we assessed LPA-induced tyrosine phosphorylation in Rh7777 cells transduced with control, LPA_1 or LPA_4 retrovirus. Western blot analysis with anti-pan phosphotyrosine antibody (Cell Signaling) was performed to detect tyrosine-phosphorylated proteins induced by treatment with different concentrations of LPA for 5 min. As shown in Fig. 2-6A, LPA induced tyrosine phosphorylation of a group of proteins of molecular weight 60-70 kD in LPA_1 and LPA_4 expressing cells, but not in control virus transduced cells. The stimulation of tyrosine phosphorylation by LPA was dose dependent with the maximal effect seen at 1μM. Since LPA induced tyrosine phosphorylation of various isoforms of paxillin with similar molecular weights (111), we examined whether the tyrosine phosphorylated proteins represent paxillin by immunoblotting with a paxillin phospho-specific antibody. As shown in Fig. 2-6B, LPA stimulated strong tyrosine phosphorylation of paxillin in LPA_1 expressing cells, but not in LPA_4 or control cells, indicating that the tyrosine phosphorylated proteins seen in LPA_4-expressing cells were not paxillin. Further, LPA_1, but not LPA_4 was capable of mediating LPA-induced paxillin phosphorylation.

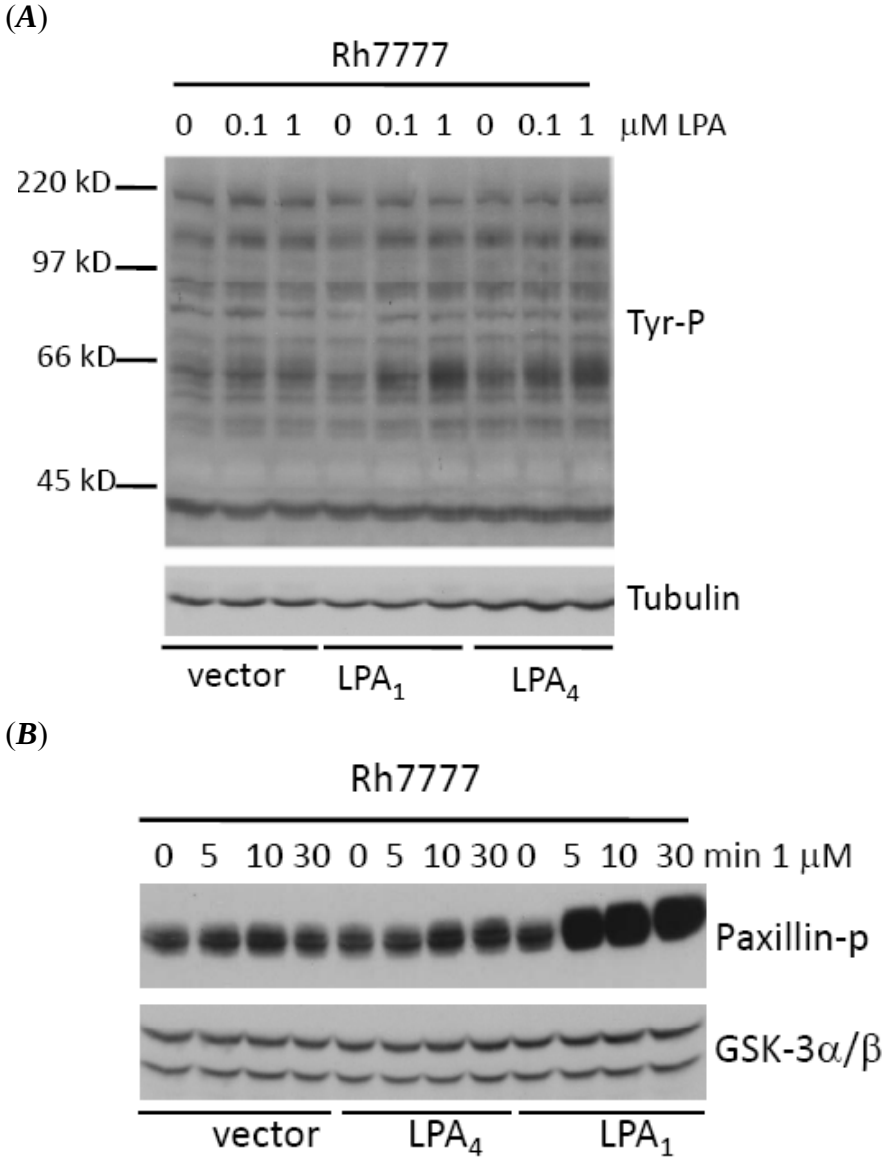


Figure 2-6. LPA_4 mediates LPA-dependent tyrosine phosphorylation in Rh7777 cells. (***A***) LPA induced tyrosine phosphorylation of 60-70 kD proteins in LPA_1 or LPA_4-expressing Rh7777 cells but not in vector control cells. The cells were stimulated for 5 min with the indicated concentrations of LPA, and analyzed by immunoblotting with an anti-phosphotyrosine antibody. (***B***) LPA_1, but not LPA_4 mediated LPA-induced paxillin phosphorylation. Control, LPA_1, or LPA_4-expressing Rh7777 cells were stimulated with

1μM LPA for the indicated periods of time (min). The cell lysates were analyzed by immunoblotting using anti-phospho paxillin antibody or anti-GSK3α/β antibody (loading control).

LPA_4 does not mediate activation of MAPK or AKT in Rh7777 cells

To obtain further evidence that LPA_4 impinges on LPA signal transduction, we examined whether LPA_4 mediates the activation of ERK and AKT as do the Edg LPA receptors. In Rh7777-LPA_1 expressing cells, LPA induced a dose-dependent phosphorylation of ERK and AKT. However, LPA at concentrations up to 10 μM did not induce AKT and ERK phosphorylation in control or LPA_4-expressing cells. Since AKT and ERK activation by LPA is G_i dependent, the data is consistent with the inability of LPA_4 to couple to G_i (54,59,60).

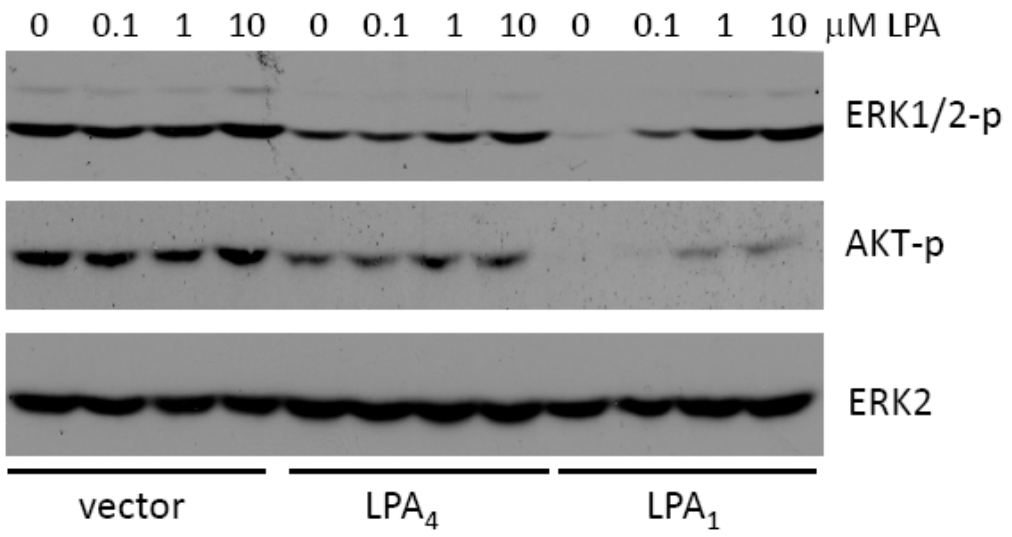


Figure 2-7. LPA_1, but not LPA_4 mediates LPA-induced ERK and AKT phosphorylation in Rh7777 cells. The vector, LPA_4, or LPA_1-expressing Rh7777 cells were grown to subconfluence, starved in serum-free medium and stimulated with LPA at indicated concentration for 5 mins. The cells were lysed and analyzed for AKT and ERK phosphorylation by immunoblotting. The total ERK was included as protein level controls.

LPA_4 mediates neurite retraction, but does not evoke migratory response to LPA in the B103 neuroblastoma cells

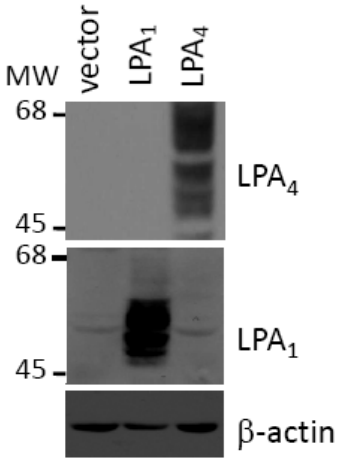
LPA induces cytoskeleton remodeling and morphological changes. In neuronal cells, LPA triggers neurite retraction and cell rounding. This response is mediated via activation of Rho by LPA receptors (5,90). Since LPA_4 couples to the $G_{12/13}$-Rho pathway, we examined the ability of LPA_4 in stimulation of neurite retraction and cell rounding in rat neuroblastoma B103 cells which do not have significant expression of endogenous LPA receptors (107). The parent cell line did not respond to LPA stimulation (60). Therefore, we overexpressed LPA_4 or LPA_1 in B103 cells by retrovirus mediated transduction as described above. The exogenous expression of LPA_4 or LPA_1 in transduced cells was confirmed by western blot (Fig. 2-8A). The predicted molecular mass for LPA_4 nd LPA_1 are both around 40-50 kDa. However, Western blotting analysis showed the presence of multiple bands specifically recognized by anti-LPA_4 antibody in LPA_4-tranduced cells, likely representing formation of aggregates and/or post-translational glycosylation of the overexpressed LPA_4.

The LPA receptor-transduced cells displayed morphologies different from each other or from control virus-infected cells (Fig. 2-8B). B103-LPA_4 cells had tendency to form aggregates particularly at relatively low cell density while expression of LPA_1 was associated with a more flattened cell shape as described previously (60,91). After incubation overnight in serum-free medium, the majority of the cells showed long neurites. Upon LPA stimulation, neurites remained to be extended in the parent or B103-vector cells upon LPA stimulation. In sharp contrast, LPA induced rapid and robust retraction of

neurites in LPA_1- or LPA_4- expressing B103 cells (Fig. 2-9). Most cells became rounded and lost neurites within minutes of LPA stimulation. As indicated in Fig. 2-9, 20-30% cells were scored round compared to less than 5% control cells showing round morphology. The data suggests that expression of LPA_4 is sufficient to induce neurite retraction in response to LPA.

We next examined whether LPA_4 mediates LPA-induced chemotactic migration using transwell chambers precoated with collagen. As shown in Fig. 2-10, LPA strongly stimulated migration of B103-LPA_1 cells but not of B103-vector or B103-LPA_4 cells. The data suggest that LPA_4 is not competent to evoke the migratory response to LPA although it was highly expressed in the transduced B103 cells.

(***A***)



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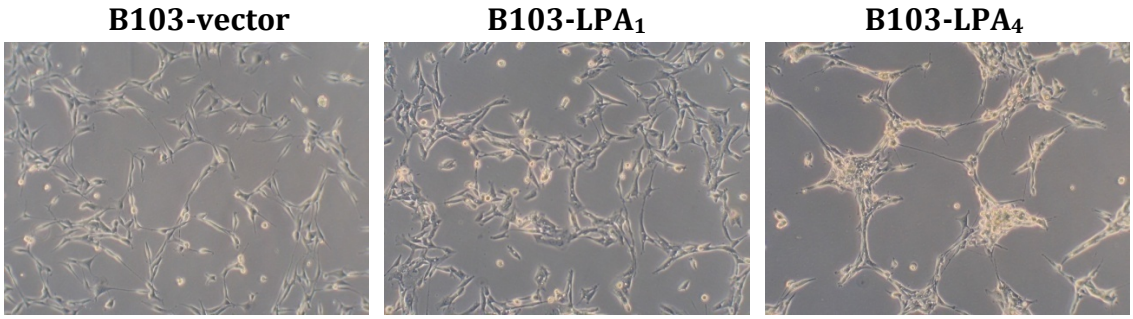


Figure 2-8. Overexpression of LPA_1 or LPA_4 in B103 cells leads to morphological changes. (***A***) B103 cells were infected with vector, LPA_1 or LPA_4-expressing retrovirus. The expression of LPA_1 and LPA_4 was confirmed by Western blotting. (***B***) Phase-contrast microscopic photographs of vector control, LPA_1 and LPA_4- expressing B103 cells.

(***A***) (***B***)

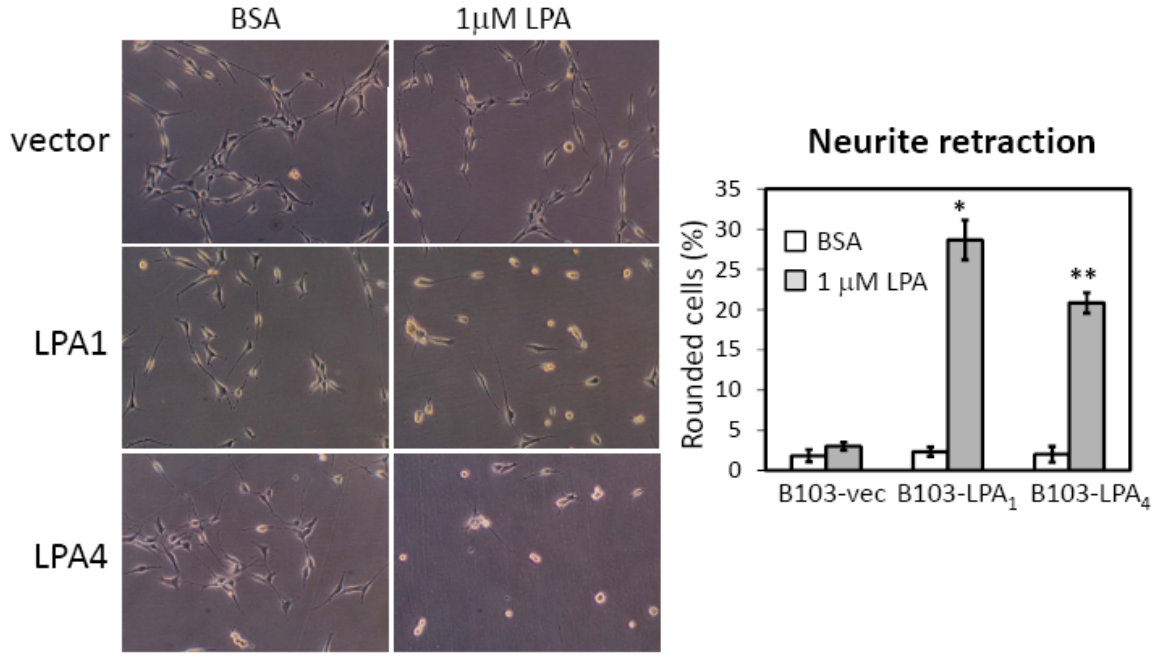


Figure 2-9. LPA_4 mediates neurite retraction in LPA-treated B103 cells. Serum-starved B103 cells (vector, LPA_1- or LPA_4-expressing) were exposed to 1 μM LPA for 15 min. LPA-induced neurite retraction was monitored under microscope (***A***) and quantified as percentages of round cells versus total number of cells (***B***). Data are the mean ± SEM (n = 3). *, $p = 0.00028$; **, $p = 0.000017$ (Student's *t* test).

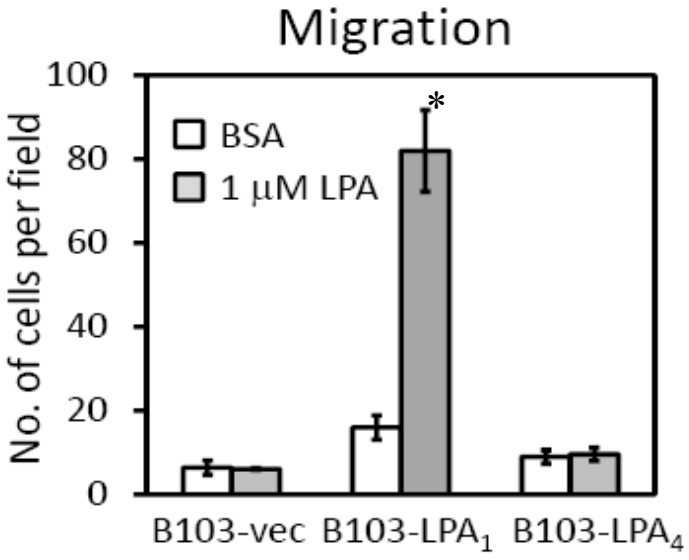


Figure 2-10. LPA_4 does not mediate LPA-induced chemotaxis. LPA-induced chemotactic migration of vector, LPA_1 or LPA_4-expressing B103 cells was analyzed with transwell chambers as detailed in Materials and Methods. The cells (1×10^4 cells/well) were loaded onto the wells and allowed to migrate toward 1μM LPA for 4 hr. The data are expressed as the average number of cells in five randomly selected fields under microscope (200 ×) ± SD of triplicate determinations, representative of three independent experiments. *, $p = 0.00035$ (Student's *t* test).

Discussion

A major advance in understanding roles of lysophospholipids has been the identification of LPA receptors that meet clear, unambiguous criteria for receptor function, combined with independent confirmation of the proposed identity. In the lysophospholipid receptor field, multiple instances of misidentification have occurred, most notably the following: OGR1 as a sphingosylphosphorylcholine receptor (102), GPR4 as a lysophosphatidylcholine and sphingosylphosphorylcholine receptor (106), G2A as an lysophosphatidylcholine receptor (104,116), and mammalian PSP24 s as an LPA receptor (105). Since the initial report of LPA_4 as a novel LPA receptor in 2003, it has been controversial whether this is indeed a functional LPA receptor mainly due to its different structure from the known Edg LPA receptors.

My assessment here of LPA_4 supports its initial identification (54). Several lines of evidence include induction of protein tyrosine phosphorylation and neurite retraction/cell rounding. These cellular responses to LPA are known to be mediated through the $G_{12/13}$-Rho signaling cascade, indicating that LPA_4 is an LPA receptor subtype coupled to the $G_{12/13}$ subunit effectively. The most definitive evidence for the identity of LPA_4 was provided by the specific binding of [^{3}H]-LPA to heterologously expressed LPA_4 present in membrane fractions. Due to the hydrophobic nature of the lysophospholipids resulting in heavy nonspecific background binding to cell membrane, the receptor-ligand binding analysis is not a straight-forward assay. We have used both LPA receptor-positive and negative cell lines to assess the binding between LPA and cell membrane fractions. In both

cell types, we were able to show increased membrane binding to LPA in the cells overexpressing exogenous LPA_4 compared to the cells not overexpressing LPA_4.

Compared to the pluropotent LPA_1 receptor, LPA_4 was not capable of mediating LPA-induced activation of ERK and AKT which are located downstream of G_i protein. The data confirms that LPA_4 does not couple to G_i subunits of trimetric G proteins. Consistent with this, LPA_4 does not mediate migratory response to LPA, a biological action of LPA dependent on G_i-mediated signaling processes. In summary, our results indicate that LPA_4 is a functional LPA receptor. Different from the LPA_1 receptor, LPA_4 mediates only a subset of cellular responses to LPA.

CHAPTER 3: GENERATION OF LPA_4/p2y9/GPR23-DEFICIENT MICE

Introduction

LPA_4, LPA_5 and LPA_6 of the purinergic receptor family are structurally distant from the Edg LPA_{1-3} receptors. The structure difference implies that LPA_4 LPA_5, or LPA_6 may possess physiological functions different from those of the Edg LPA receptors. However, little is known about the expression and functions of these newly identified LPA receptors. Targeted deletion is one of the most relevant models to examine the biological roles of genes of interest. It has become an important approach to probe functions of the Edg LPA_{1-3} receptors in embryonic development, health and diseases. Although initial analysis revealed only minor phenotypic changes associated with loss of individual LPA receptors, more extensive studies of these animals have elucidated unexpected roles for LPA_1 in the development of vascular and nervous systems (63), initiation of neuropathic pain (66), and promotion of pulmonary and renal fibrosis (64,65). Thus it is critically important to develop a knockout mouse model to investigate *in vivo* functions of LPA_4 and other new LPA receptors.

In this part of the study, we disrupted the LPA_4-encoding gene (*lpa_4/p2y9/gpr23)* by targeted deletion in mice. Similar to knockout of *lpa_1* or *lpa_2* (63,79), LPA_4-deficient

mice did not show obvious abnormalities in embryonic development, fertility or normal physiology. However, the availability of the mice and LPA_4-null tissues and cells has allowed us to analyze phenotypic alterations in cell signaling and responses to LPA, which will be discussed in detail in Chapter 3.

Materials and Methods

Generation of lpa_4 targeting vector

The genomic sequences of the mouse *lpa_4* (C57BL/6) were isolated and PCR amplified from a BAC clone (RP23-343P30) (BACPAC Resources, Oakland, CA). A 1.965-kb *KpnI/EcoRI* fragment containing the 3' part of exon 3 was PCR amplified from the BAC DNA and cloned as the short arm into the pKO Scrambler NTKV-1901 targeting vector that carries both PGK/*neo*/BGH cassette for positive selection of homologous recombinants with G418 and an MC1-*tk* cassette for negative selection of non-homologous recombinants with gangcyclovir (Stratagene, La Jolla, CA). The 5.34-kb *EcoRI/BstBI* fragment containing exon 2 and the major part of intron 2 was cloned into pBluescript II SK (+) from the BAC DNA and recloned using the *Not I* and *Sal I* sites into the targeting vector. Thus, a 2.591-kb fragment containing the 3' end of intron 2 and the 5' portion (with the complete coding sequence) of exon 3 was replaced with 1.603 kb of the PGK/*neo*/BGH cassette, creating the final targeting vector (Fig. 3-2A).

Generation of LPA_4-deficinet mice

All procedures for animal studies were conducted in compliance with the policies and regulations of Virginia Commonwealth University IACUC. The *lpa$_4$* targeting vector was linearized with *Not I* and electroporated into the *129/Sv* embryonic stem (ES) cells HZ2.2 (generated by the VCU Transgenic/Knockout Mouse Core). Genomic DNA from 82 ES cell clones resistant to both G418 and gangcyclovir were screened for homologous recombination by long-range PCR using an *lpa$_4$* intron 1 sense primer (2717-2740: 5'-CCAAATGTAGGTGCCACTTGTATG-3') and a PGK anti-sense primer (5'-GGTGGATGTGGAATGTGTGCGAG-3') for the long arm, and a *neo* sense primer (5'-TCGCCTTCTTGACGAGTTCTTCTG-3') and a 3' flanking anti-sense primer (12725-12702: 5'-GCCTACAGCCTTATGTATTCCAAC-3') for the short arm. Southern blot analysis was performed on DNA from these clones using a PGK*/neo/*BGH probe to verify clone purity and the integrity of both genomic arms. Two independent recombinant clones were injected into *C57BL/6* blastocysts which were then implanted into pseudopregnant *CD-1* recipients, producing two independent lines of chimeric male mice. They were bred with *C57BL/6* females to generate heterozygous female founder mice (X^+X^-) and wild type WT males (X^+Y), which were intercrossed to generate more heterozygous females (X^+X^-) and hemizygous KO males (X^-Y). These WT, heterozygous and hemizygous mice were further crossed to produce all genotypes summarized in Table 1.

Genotyping of lpa$_4$ KO mice

Genotyping for *lpa$_4$* alleles were done by PCR amplification of mouse tail genomic DNA. Mouse tail biopsies (0.5 cm) were dissolved in DirectPCR Lysis Reagent (VIAGEN

Biotech, CA) containing 0.2 mg/ml proteinase K at 65°C overnight. The crude lysates were incubated in 100°C heat block for 5 min to inactivate residual proteinase K. Mouse hairs and undigested tissues were precipitated by brief centrifugation. The supernatants containing mouse genomic DNA were used as templates for PCR amplification of genomic DNA (40 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min) using a common *lpa*$_4$ intron 2 sense primer (7713-7735: 5'-CTATTGCTTTCCCCCATGTTATG-3'), an *lpa*$_4$ intron 2 anti-sense primer (8107-8084: 5'-TTACTATTGGCTAGTCTGTCTTTC-3') for the WT allele, and a PGK anti-sense primer (5'-GGTGGATGTGGAATGTGTGCGAG-3') for the KO allele (Fig. 3-2C). Expected sizes of the PCR products for WT and KO alleles were 395 and 564 bp, respectively. The line was subsequently maintained in a mixed *129/Sv* and *C57BL/6* background.

RT-PCR analysis of LPA$_4$ mRNA

Total RNA was isolated from mouse tissues using TriZol according to the manual instruction (Invitrogen). RNA (1 μg) was transcribed to cDNA usingthe ThermoScript™ RT-PCR kit (Invitrogen) with random hexamer primers. LPA$_4$ cDNA was amplified by PCR using primers: forward 5'- GTGCGAGTTGCCAGTTTACACGTT-3' and reverse 5'-ACTGATGCAGGTGAGGAAGAGCAT-3'. The primers for amplification of LPA$_1$ and LPA$_2$ are LPA$_1$ forward primer: 5'-ACTGACTGTTAACACGTGGCTCCT-3' and reverse primer 5'-GGCAGCACACATCCAGCAATAACA-3'; LPA$_2$ forward primer 5'-ACTGCCTCTGTGACTTGGACAGTT and reverse primer 5'-AAGCTGAGTAACGGGCAGACTTGT. GAPDH was used as control. Reaction

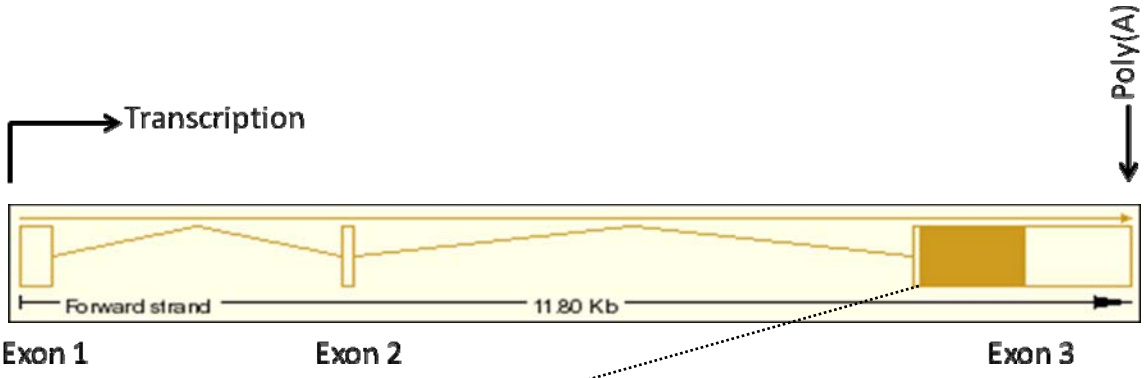
conditions were optimized to obtain amplification within the logarithmic phase of the reaction.

Results

Development of LPA$_4$-deficient mice

Similar to the human *lpa$_4$*, the murine *lpa$_4$* gene are located on the X chromosome (chrX: 98, 325, 012-98, 336, 788) (Gene ID: 78134). Unlike its intronless human homolog, the mouse *lpa$_4$* has 3 exons spanning approximately 11.8 Kb while the coding sequence is located within exon 3 only. The coding sequence contains an open reading frame of 1113 base pairs encoding a protein of 370 amino acid residues with a deduced molecular weight of 41.89 kDa. To disrupt the *lpa$_4$* locus, we replaced the 3' end of intron 2 and 5' portion of exon 3 that contains the full coding sequences of LPA$_4$ with the PGK/*neo*/BGH cassette. As detailed in Materials and Methods, the chimeric male mice were bred with *C57BL/6* females to generate heterozygous female founder mice (X^+X^-) and WT males (X^+Y), which were further intercrossed to generate hemizygous males (X^-Y). The genotypes of the offspring on the mixed C57BL/6 and 129/Sv background were identified by PCR amplification of genomic DNA obtained from tails of mice.

(***A***)



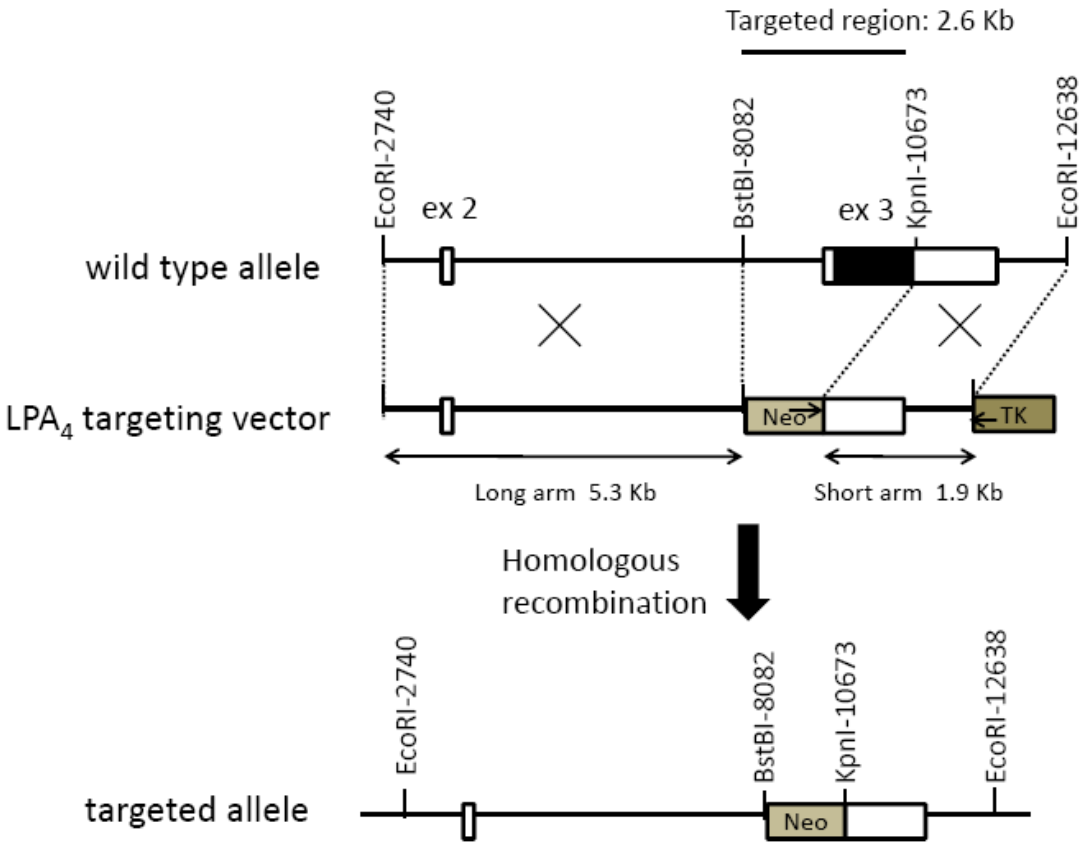
(***B***)

```
  1 ATGGGTGACAGAAGATTTATTGACTTCCAATTCCAAGATTTAAATTCAAGTCTCAGACCC 60
    M  G  D  R  R  F  I  D  F  Q  F  Q  D  L  N  S  S  L  R  P

 61 AGGTTGGGAAATGCAACTGCCAATAATACTTGCATTGTTGATGATTCCTTCAAGTATAAT 120
    R  L  G  N  A  T  A  N  N  T  C  I  V  D  D  S  F  K  Y  N

121 TTGAATGGTGCTGTCTATAGTGTTGTATTCATCCTGGGTCTAATAACCAGCAGTGCCTCC 180
    L  N  G  A  V  Y  S  V  V  F  I  L  G  L  I  T  S  S  A  S
                                  TMD I
181 CTGTTTGTCTTCTGCTTCCGCATGAAAATGAGAAGTGAGACGGCTATTTTCATCACCAAC 240
    L  F  V  F  C  F  R  M  K  M  R  S  E  T  A  I  F  I  T  N

241 CTGGCCCTCTCTGATTTGCTTTTTGTTTGTACCCTACCTTTCAAAATATTTTACAACTTT 300
    L  A  L  S  D  L  L  F  V  C  T  L  P  F  K  I  F  Y  N  F
                TMD II
301 AATCGCCACTGGCCTTTTGGTGACACCCTCTGTAAGATCTCAGGGACTGCGTTCCTCACC 360
    N  R  H  W  P  F  G  D  T  L  C  K  I  S  G  T  A  F  L  T

361 AACATCTATGGGAGCATGCTCTTCCTCACCTGCATCAGTGTGGATCGTTTCCTAGCCATT 420
    N  I  Y  G  S  M  L  F  L  T  C  I  S  V  D  R  F  L  A  I
             TMD III
421 GTCTATCCCTTCCGATCGCGTACCATCAGGACCAGGAGGAATTCCGCCATTGTGTGCGCT 480
    V  Y  P  F  R  S  R  T  I  R  T  R  R  N  S  A  I  V  C  A
481 GGAGTCTGGATCCTAGTCCTCAGTGGTGGTATTTCAGCTTCTTTGTTCTCCACCACTAAT 540
    G  V  W  I  L  V  L  S  G  G  I  S  A  S  L  F  S  T  T  N
             TMD IV
541 GTCAACAATGCGACCACCACTTGCTTTGAAGGCTTCTCCAAACGTGTCTGGAAGACATAC 600
    V  N  N  A  T  T  T  C  F  E  G  F  S  K  R  V  W  K  T  Y

601 CTGTCCAAGATCACTATATTCATTGAAGTTGTTGGATTCATCATTCCTCTGATATTGAAT 660
    L  S  K  I  T  I  F  I  E  V  V  G  F  I  I  P  L  I  L  N
                                  TMD V
```

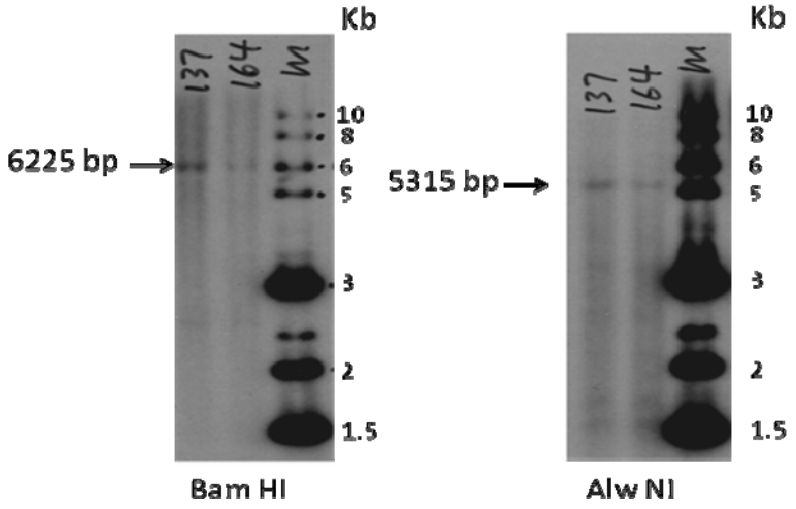
```
 661 GTTTCTTGTTCTTCTGTGGTGCTTAGAACCCTCCGCAAGCCTGCAACATTGTCTCAGATT 720
      V  S  C  S  S  V  V  L  R  T  L  R  K  P  A  T  L  S  Q  I

 721 GGGACCAATAAAAAAAAAGTGTTGAAGATGATCACAGTGCATATGGCAGTGTTTGTGGTA 780
      G  T  N  K  K  K  V  L  K  M  I  T  V  H  M  A  V  F  V  V

 781 TGCTTTGTACCATACAACTCCGTTCTCTTTTTATATGCCTTGGTACGCTCCCAAGCCATT 840
      C  F  V  P  Y  N  S  V  L  F  L  Y  A  L  V  R  S  Q  A  I
        TMD VI
 841 ACTAATTGCTTATTGGAAAGGTTTGCAAAGATCATGTACCCAATTACCTTGTGCCTTGCA 900
      T  N  C  L  L  E  R  F  A  K  I  M  Y  P  I  T  L  C  L  A
                                                  TMD VII
 901 ACTCTGAATTGTTGCTTTGATCCTTTTATCTATTACTTCACTCTTGAATCCTTTCAGAAG 960
      T  L  N  C  C  F  D  P  F  I  Y  Y  F  T  L  E  S  F  Q  K

 961 TCCTTTTATATCAATACACATATAAGGATGGAGTCGCTGTTTAAGACTGAGACACCTCTG 1020
      S  F  Y  I  N  T  H  I  R  M  E  S  L  F  K  T  E  T  P  L

1021 ACCCCCAAACCTTCCCTTCCAGCTATCCAAGAGGAAGTTAGTGATCAAACAACAAATAAT 1080
      T  P  K  P  S  L  P  A  I  Q  E  E  V  S  D  Q  T  T  N  N

1081 GGTGGTGAATTAATGCTGGAATCCACCTTCTAG 1113
      G  G  E  L  M  L  E  S  T  F  *
```

Figure 3-1. Diagrammatic representation of the genomic structure of mouse *lpa*$_4$ gene and coding sequences with its putative seven transmembrane domains. (***A***) Schematic representation of the domain structures of the mouse *lpa*$_4$ gene. (***B***) Coding sequences and corresponding amino acids.

(***A***)


Targeted region: 2.6 Kb
EcoRI-2740
ex 2
BstBI-8082
ex 3
KpnI-10673
EcoRI-12638
wild type allele
LPA4 targeting vector
Neo
TK
Long arm 5.3 Kb
Short arm 1.9 Kb
Homologous recombination
EcoRI-2740
BstBI-8082
KpnI-10673
EcoRI-12638
targeted allele
Neo

(***B***)



(***C***)

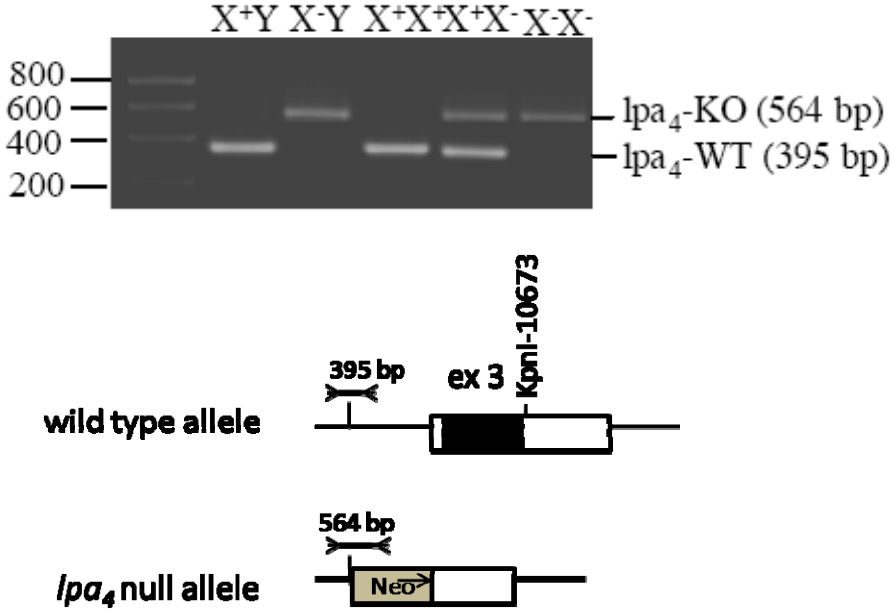
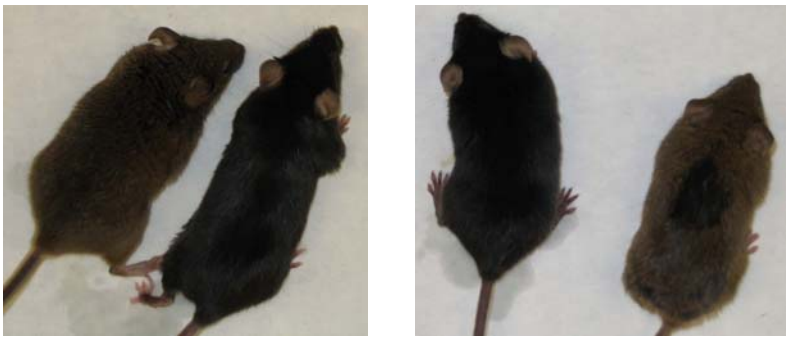


Figure 3-2. Generation of LPA$_4$-deficient mice. (***A***) Schematic representations of the wild type *lpa$_4$* allele, targeting vector, and the targeted allele are shown. The positions of

neo and *tk* genes, restriction sites and exon 2 and 3 are indicated. The targeting vector pKO Scrambler NTKV-1901 containing the *neo* gene was used to replace the 3' portion of intron 2 and the 5' fragment of exon 3 which carries the entire coding sequence of LPA_4. The positions of primers for long chain PCR of long arm and short arm detection are indicated by the arrowheads. (***B***) Genomic DNA prepared from two independent ES cell clones was subjected to Southern blot analysis after digestion with Bam HI or Alw NI restriction enzymes. A DNA fragment generated from sequence within *PGK/neo* gene was used as probe. Bands corresponding to the lpa_4 null allele with each digestion are indicated. (***C***) Genotyping by PCR amplification of genomic DNA obtained from mouse tails confirmed the DNA arrangements consistent with X^+Y, X^-Y, X^+X^+, X^+X^-, and X^-X^- genotypes. The relative locations of 3 primers used for PCR detection of wild type and KO alleles are indicated by the arrowheads. The product size of 395 bp and 564 bp represent the wild type and KO alleles, respectively.

Characterization of lpa_4 heterozygous and homozygous mice

As demonstrated in Table 1, the gender ratios of the offspring [male/(male+female)] from $X^+Y \times X^+X^-$ crossing and from $X^-Y \times X^+X^-$ crossing were 43.5 % and 46.6 %, respectively with an overall percentage of 45.1 %, slightly lower than the expected 50%. The differences, however, lacked statistical significance when examined with the chi-square test of goodness-of-fit. In addition, heterozygous females (X^+X^-), homozygous females (X^-X^-) and hemizygous males (X^-Y) were born at statistically expected Mendelian rule, reflecting that loss of LPA_4 does not cause embryonic lethality or impose a detrimental effect on embryonic development (Table 1). The *lpa4* KO mice were grossly indistinguishable from their WT or heterozygous littermates in appearance, size, and behavior (Fig. 3-3). They did not show any defects in mating, pregnancy, or litter sizes. There were no gross abnormalities in the internal organs of LPA_4-deficient adults (data not shown). Some LPA_4-deficient mice have survived 18 months, indicating that the loss of LPA_4 does not shorten life span of mice.



WT KO WT KO

Figure 3-3. Pictures of *lpa*$_4$ WT and KO mice.

Table 1. Inheritance of the *lpa*$_4$ deletion allele

	No. of offspring with *lpa*$_4$ genotypes						
	males		females				
Crossing pairs	X^+Y	X^-Y	X^+X^+	X^+X^-	X^-X^-	Sex ratio m:f (% m/m+f)	Total No. of mice

$X^+Y \times X^+X^-$	24 (17.4)	36 (26.1)	40 (29.0)	38 (27.5)	0 (0)	60:78	(43.5)	138
$X^-Y \times X^+X^-$	33 (24.8)	29 (21.8)	0 (0)	41 (30.8)	30 (22.6)	62:71	(46.6)	133

Numbers of individual progeny genotyped from the indicated crosses are shown. In parentheses are the percentages of the mice with the indicated genotypes or percentages of males versus the total numbers of mice generated from the indicated crosses.

Expression of LPA_4 in mice tissues

To delineate the tissue distribution of LPA_4, we examined its mRNA expression in a number of adult tissues including the liver, heart, skeleton muscle, rectum, kidney, lung and ovary, uterus or testis. As analyzed by RT-PCR, LPA_4 mRNA transcript was present in

the heart, skeleton muscle and ovary but weakly seen in the liver or testis (Fig. 3-4A). Further analysis of more tissues of adult mice indicates that LPA_4 mRNA was present in the lung, kidney, and rectum (Fig. 3-4B). The expression of LPA_4 mRNA in these tissues was absent from homozygous females and hemizygous males further confirming loss of LPA_4 in *lpa4* KO mice. The expression of LPA_1 transcripts in the these tissues among wild type, heterozygous, and knockout mice was not significantly changed, suggesting that loss of LPA_4 does not lead to compensatory increases in expression of other LPA receptors.

(***A***)

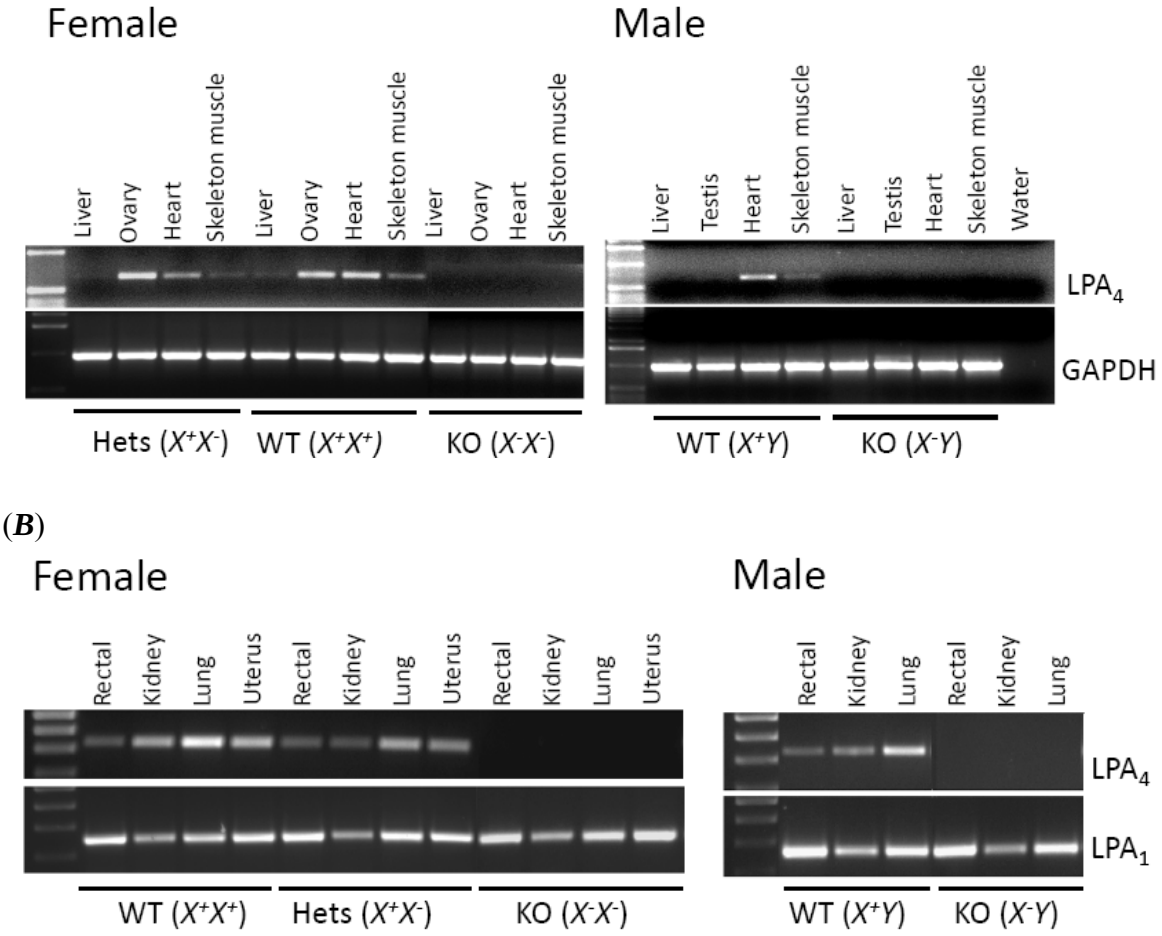


Figure 3-4. Homozygous deletion of *lpa₄* in KO mice and the expression patterns of LPA_4 in adult mice tissues. RNA was extracted from adult mouse tissues and reverse transcription-PCR was performed with 27 cycles (***A***) or 35 cycles (***B***) amplification. The products were separated on agarose gels and visualized with ethidium bromide staining. RT-PCR amplification of GAPDH or LPA_1 was included as controls.

Discussion

To understand the physiological functions of the LPA_4 receptor, we have disrupted its encoding gene in mice by targeted deletion. The *lpa4* KO mice were born at expected Mendelian frequency without sexual bias. The adult mice were physiologically normal and were grossly indistinguishable from their WT siblings in appearance, size, and behavior, suggesting that LPA_4 is not required for embryonic development and normal physiology. This is similar to targeted deletion of *lpa1* or *lpa2* (63,79). One plausible explanation for the lack of prominent phenotypes in LPA_4-deficient mice is that other backup and/or redundant receptor subtypes of LPA may suffice to compensate for the loss of these LPA receptors *in vivo*. In particular, the recently identified LPA_5 and LPA_6 receptors are closely related to LPA_4. Alternatively, LPA, as one of phospholipids present in the circulation and tissues, may not be only or rate-limiting mediator physiologically required *in vivo*. LPA signaling may be more critical in pathophysiological settings when levels of the lipid mediator are locally and temporally altered. In light of the pathophysiological significance of LPA signaling, it will be interesting to study the LPA_4-deficient mice in stress such as tissue injury, inflammation or exposure to chemical carcinogens, which may reveal novel functions of the receptor. Recent studies of LPA_1-deficeint mice subjected to injury demonstrated the involvement of LPA_1 in abnormal wound healing and fibrosis formation (64-66), consistent with a major role of this LPA receptor in chemotactic recruitment of fibroblasts to the site of wound.

The availability of the *lpa4* KO mice makes it possible to generate mice lacking multiple isoforms of LPA receptors to address the redundancy and complexity of LPA signaling. Furthermore, LPA_4-deficient mice provide us a unique opportunity to study the

functional consequence of loss of LPA_4 by using truly LPA_4–negative tissues and cells. We have obtained such results that suggest a role of LPA_4 in antagonism of the Edg LPA receptors as will be discussed in Chapter 4.

CHAPTER 4: LPA_4/p2y9/GPR23 IN NEGATIVE REGULATION OF CELL MOTILITY

Introduction

Stimulation of cell motility is one of the major biological effects of LPA and its producing enzyme ATX. LPA-induced cell movement is mediated mainly by LPA_1, although LPA_2 or LPA_3 may also capable of evoking the response to a lesser degree in various cellular contexts (3,11,36,62,117,118). However, little is known about how the migratory response to LPA is appropriately controlled in mammalian cells that usually coexpress multiple LPA receptor subtypes endogenously. The *lpa4* KO mice we have developed provide a valued cellular model to analyze the migratory response to LPA in the absence of a specific receptor. The results of the experiments using LPA_4-deficient cells and other cellular models establish a novel role of LPA_4 in the negative control of LPA-mediated cell migration and invasion. We demonstrated that LPA_4-deficient mouse embryonic fibroblasts (MEFs) from *lpa4* KO mice were hypersensitive to LPA-induced migration. Consistent with negative modulation of the phosphatidylinositol 3 kinase pathway by LPA_4, LPA_4 deficiency potentiated AKT and Rac but decreased Rho activation induced by LPA. Reconstitution of LPA_4 converted LPA_4-negative cells into a less motile phenotype. In support of the biological relevance of these observations, ectopic expression of LPA_4 strongly inhibited migration and invasion of human cancer cells. When coexpressed with LPA_1 in B103 neuroblastoma cells devoid of endogenous LPA receptors,

LPA_4 attenuated LPA_1-driven migration and invasion, indicating functional antagonism between the two subtypes of LPA receptors. These results provide genetic and biochemical evidence that LPA_4 is a suppressor of LPA-dependent cell migration and invasion in contrast to the motility-stimulating Edg LPA receptors.

Materials and Methods

Reagents

Protease inhibitor cocktail tablets and BSA were purchased from Roche (Indianapolis, IN). Plasmid DNA was purified using the endo-free purification kit from Qiagen (Valencia, CA). All oligonucleotides were synthesized by Operon Biotechnologies, Inc (Huntsville, AL). TRIzol and cell culture medium were obtained from Invitrogen Inc. (Carlsbad, CA). Fetal bovine serum (FBS) was from Biomeda (Foster City, CA). Epidermal growth factor (EGF), Ki16425, and anti-flag M2 and anti-ß-actin monoclonal antibodies were obtained from Sigma (St. Louis, MO). Pertussis toxin (PTX) was purchased from List Biological Laboratories, Inc. (Campbell, CA). AKT inhibitor II (AKTi II) was obtained from Calbiochem (San Diego, CA). Anti-phospho AKT, anti-phospho ERK and anti-tubulin antibodies, and LY294002 were obtained from Cell Signaling (Beverly, MA). The rabbit polyclonal antibody against the C-terminus of the human LPA_4 was kindly provided by Dr. T Shimizu (University of Tokyo).

MEFs

MEFs were isolated from E12.5–13.5 embryos essentially as described (120). The embryos were separated from the uterine wall and amniotic sac and placed in Petri dishes containing small volumes of PBS. The head and heart tissues were removed. The remaining embryo were minced with surgical scissors and razor blade, digested with 0.25% trypsin/EDTA at 37 ^{o}C for 25 min, and triturated through a Pasteur pipette. After digestion and removal of undigested tissues, the cells were spun briefly and plated into a 10-cm dish and allowed to grow to subconfluence in DMEM+10% FBS. The cells were then either frozen as passage 1 or subcultured at a 1:4 ratio for experiments. Immortalized cell lines were established from primary MEFs using standard protocols for generation of 3T3 cells (121).

The genotypes of MEFs were determined by PCR amplification of genomic DNA isolated from MEFs as described in Chapter 3. The gender of embryos was determined by PCR amplification of the *SRY* (Sex-determining Region Y gene) locus with a SRY-sense primer (5'-ATTTATGGTGTGGTCCCGTGGTGA-3') and a SRY anti-sense primer (5'-TTGCCTGTATGTGATGGCATGTGG -3'). The size of the PCR product for *SRY* was 313 bp.

Other cells

The DLD1 colon cancer cell line was kindly provided by Dr. D Shida (Virginia Commonwealth University) and maintained as described (72). The B103 rat neuroblastoma cell line lacking endogenous LPA receptors was obtained from Dr. J. Chun (Scripps

Research Institute) and cultured in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin (107). The rat hepatoma cell line Rh7777 was purchased from ATCC and maintained in the same conditions as B103. These cell lines were frozen at early passages and used for less than 6 weeks in continuous culture.

RT-quantitative PCR

cDNA was synthesized from 1 μg RNA using the ThermoScript™ RT-PCR kit (Invitrogen) with random hexamer primers. Quantitative PCR was performed with the pre-mix TaqMan primer sets on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems, CA).

Recombinant retroviruses and infection of cells

The Human LPA_4 or LPA_1 cDNA was inserted between *Bam H I* and *Xho I* sites upstream of the internal ribosomal entry site of the Moloney murine leukemia retrovirus vector pLZRS-EGFP (a gift of J. Chun, Scripps Research Institute) (107). The structure and sequences of the cDNAs in these viral constructs were confirmed by restriction digestion and automatic sequencing. The Bosc23 packaging cell line (ATCC) was transfected with pLZRS-EGFP, pLZRS-EGFP-LPA_4 or pLZRS-EGFP-LPA_1 using Lipofectamine 2000 as described (107). Approximately 20 hours after the beginning of transfection, the cells were fed fresh DMEM+10% FBS. Culture supernatants containing retrovirus were harvested 48 hours late, cleared by centrifugation and used to infect cells or stored at –80°C.

The LPA_1 and LPA_4 cDNAs were also cloned into the pLenti-TOPO lentivirus vector (Invitrogen) as an alternate expression system for LPA receptors. The pLenti-TOPO-LPA_1, pLenti-TOPO-LPA_4 or pLenti-TOPO-LacZ vector was transfected along with packaging plasmids into 293FT cells (Invitrogen) to replicate lentivirus using a protocol similar to that for retrovirus generation in Bosc23 cells. MEFs and other cell lines in 35-mm dishes at around 50% confluence were incubated for 16-22 hours with 1.5 ml of viral supernatants containing 8 μg/ml of Polybrene. The infected cells were harvested 72 hours after infection. For the cells infected with the retrovirus, EGFP-positive cells were isolated by fluorescence-activated cell sorting (FACS). Lentivirus-infected cells were selected with blasticidin (10 μg/ml) for 10-14 days and pooled colonies were expanded for experiments. When co-expression of LPA_1 and LPA_4 was desired, the recipient cells were infected with the pLenti-TOPO-LPA_1 lentivirus followed by infection with the pLZRS-EGFP-LPA_4 retrovirus.

Western Blot

Cells were lysed in SDS sample buffer or in ice-cold X-100 lysis buffer [1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 100 mM NaF, 10 mM Na PPi, and protease inhibitor cocktail]. Total cellular proteins were resolved by SDS-PAGE, transferred to Immun-Blot membrane [poly(vinylidene difluoride)] (BIO-RAD, Hercules, CA), and immunoblotted with antibodies following the protocols provided by manufacturers. Immunocomplexes were visualized with an enhanced chemiluminescence detection kit (Amersham, Piscataway,

NJ), using horseradish peroxidase–conjugated secondary antibodies (Cell Signaling, Danvers, MA).

Chemotaxis

Cell migration was measured in Transwell chambers (BD biosciences, pore size 8 μm). Transwells were coated with 10 μg/ml collagen 1, and placed in the lower chamber containing serum-free DMEM supplemented with LPA or EGF. Cells suspended in serum-free DMEM containing 0.1% fatty acid-free BSA were added to the upper chamber at 2.5×10^4 cells/well or 1×10^4 cells/well as indicated. Cells were allowed to migrate for 4 hr or 6 hr at 37 °C. Non-migrated cells were removed from the top filter surface with a cotton swab. Migrated cells attached to the underside of the transwells were washed with PBS and stained with crystal violet and counted under a microscope. The invasion of tumor cells were measured using Transwells coated with growth factor-reduced Matrigel Basement Membrane Matrix (pore size 8 μM, BD biosciences). The assays were performed as migration assays except that the cells were incubated for 20-24 hours before termination of the experiments.

Generation of GST-Rhotekin-RBD and GST-PAK-PBD fusion proteins

The cDNA fragments corresponding to the Rho-binding domain (RBD, residue 7-89) of the mouse Rhotekin (122) or the p21-binding domain (PBD, residues 67-150) of the human p21-activated kinase (PAK-1) (123) were cloned between the BamH I and EcoR I sites of the pGEX-2T vector (Pharmacia). The plasmids were then transformed into E. coli

BL21 to generate GST-Rhotekin-RBD and GST-PAK-PBD fusion proteins. BL21 cells transformed with the GST-PAK-PBD construct or transformed with the GST-Rhotekin-RBD construct were grown at 37 °C and 25 °C, respectively, to the logarithmic phase (A_{600}=0.5). Expression of Rhotekin-RBD and PAK-PBD fusion proteins were induced by addition of 0.25 mM and 0.5 mM isopropylthiogalactoside for 6 or 3 hours, respectively. Cells were pelleted, resuspended in Rhotekin lysis buffer (50 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 0.2 mM Na_2S_2O, 10% glycerol, 20% sucrose, 0.5 mM PMSF, 2 mM dithiothreitol, 20 μg/ml leupeptin, 80 μg/ml benzamidine, and 40 μg/ml aprotinin) or PAK lysis buffer (1% triton, 1 mM EDTA, 1mM PMSF, 15 μg/ml leupeptin, 15 μg/ml aprotinin in PBS), and sonicated. Cell lysates were centrifuged at 4°C for 20 min at 7,100 *g* and the supernatants were incubated with glutathione-Sepharose beads for 1 hour at 4°C. Protein bound to the beads was washed three times with lysis buffer and the amounts of bound GST-fusion proteins were measured by photospectometry and the integrity of the proteins was confirmed by Coomassie-stained SDS-PAGE gels.

Rho and Rac Activation Assays

Activation of Rho and Rac was analyzed by GST pulldown assays (122,123). The cells were grown in 10-cm dishes to subconfluence, starved overnight and stimulated with LPA or vehicle for the indicated periods of time. The cells were lysed in Magnesium-containing Lysis Buffer (MLB) (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% NP40, 10% glycerol, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 mM NaF). Clarified lysates were incubated for 1 hr at 4 ^{o}C with

GST-Rhotekin-RBD or GST-PAK-PBD immobilized on glutathione-coupled Sepharose beads. Beads were washed in MLB 3 times, eluted with 2 × SDS sample buffer, and analyzed by western blotting using monoclonal anti-Rac antibody (BD Biosciences, Cat No. 610650) or rabbit anti-RhoA antibody (Santa Cruz Biotechnology, SC-418).

Statistics

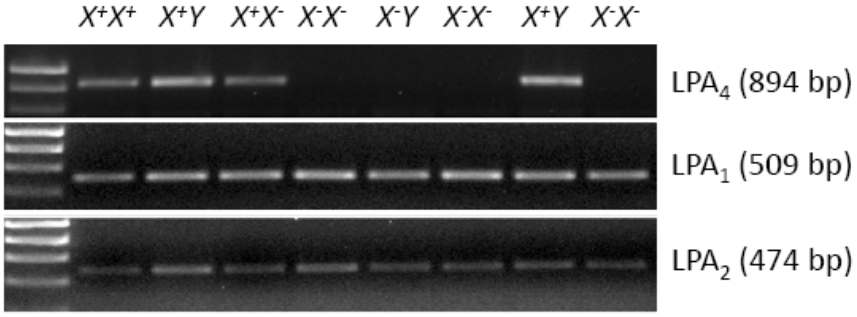
Numerical results from chemotaxis and invasion experiments were presented as average cell numbers ± SD. The statistical significances of differences were analyzed using Student's *t*-test where $P< 0.05$ was considered significant.

Results

Expression of LPA_4 and other LPA receptors in MEFs

To understand the biological functions of LPA_4, we utilized MEFs derived from E12.5 to E13.5 embryos to investigate the impact of LPA_4 deficiency on cellular responses to LPA. We first examined expression of each of LPA receptors in MEFs. RT-PCR analysis shows that wild-type MEFs expressed transcripts of LPA_1, LPA_2, and LPA_4 (Fig.4-1A), but lacked LPA_3 or LPA_5 mRNA (data not shown). As expected, no LPA_4 transcript was detected in MEFs from X^-X^- or X^-Y embryos. In addition, deletion of *lpa4* did not lead to any major compensatory changes in expression levels of LPA_1 or LPA_2 mRNA in MEFs as analyzed by RT-PCR and confirmed by RT-qPCR (4-1B).

(***A***)



(***B***)

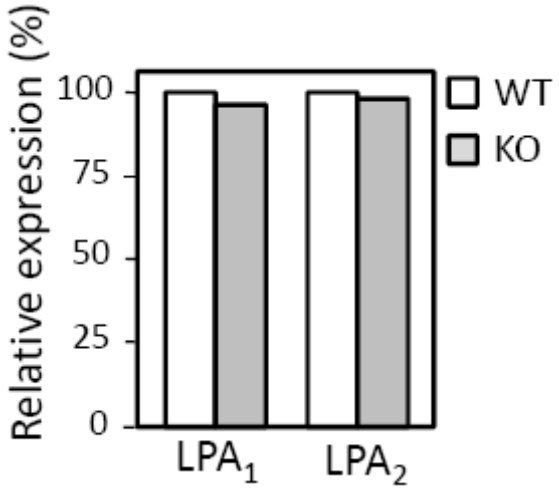


Figure 4-1. **Expression of LPA_4 mRNA in MEFs.** (***A***) Primary MEFs were prepared from the 12.5-13.5 embryos. The genotypes of MEFs were determined by PCR as described in chapter 3 and the genders determined by PCR amplification of a Y chromosome-linked locus *SRY*. The expression of each LPA receptor mRNA in MEFs was analyzed by RT-PCR, which was consistent with the wild type (X^+X^+, X^+Y), knockout (X^-X^-, X^-Y) or heterozygous (X^+X^-) genotypes. The size of PCR products was labeled in the brackets (bp). (***B***) RT-qPCR results of LPA_1 and LPA_2 mRNA in MEFs.

Distinct morphology of lpa$_4$ KO MEFs

Both LPA_4-negative and positive MEFs showed morphological characteristics of fibroblasts. However, the loss of LPA_4 in MEFs changed the cell morphology into a more spread and enlarged shape compared to wild type cells (Fig. 4-2). The striking difference in morphology between WT and KO MEFs remain evident after MEFs become immortalized in culture (Fig. 4-2).

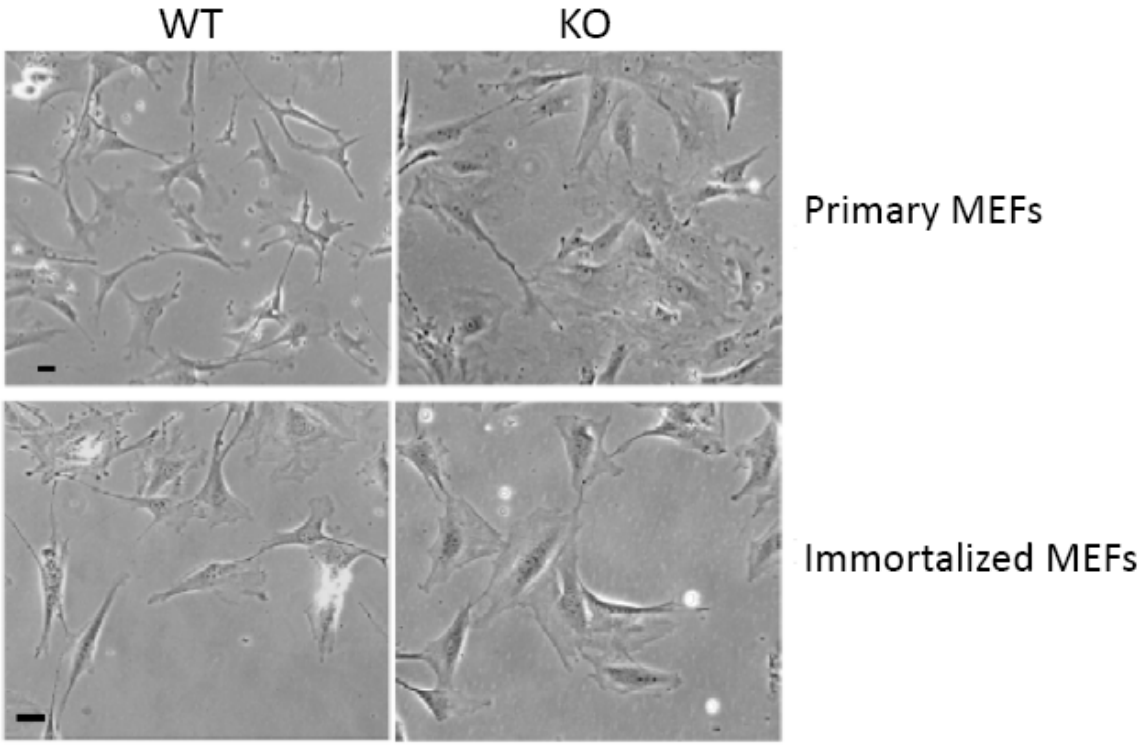


Figure 4-2. Morphology of WT and *lpa$_4$* KO MEFs. Phase contrast microscopic photographs of WT and *lpa$_4$* KO MEFs. LPA$_4$-deficient MEFs (KO2.5) showed a more flattened morphology compared to WT cells (WT1.3). The striking morphological difference was observed in both primary MEFs (*upper panels*) and immortalized MEF lines (*lower panels*). *Bar*, 5 μm.

Loss of LPA_4 potentiates cell migration to LPA, but not to EGF

Cell morphology and motility are coordinately regulated by LPA signaling through intracellular small Rho GTPases (126). The morphological alteration in LPA_4 null MEFs prompted us to ask whether loss of LPA_4 has effect on cell migration, a hallmark cellular response to LPA. We compared WT and LPA_4 null MEFs for LPA induced cell migration using chemotaxis transwell assay. Surprisingly, the LPA_4-deficient MEFs exhibited remarkably enhanced chemotactic response to LPA (14 fold increase over unstimulated cells) (Fig. 4-3) compared to WT cells that only modestly responded to LPA (2.7 fold increase). Of interest, the LPA_4-defieicent MEFs were also more motile than WT MEFs in unstimulated conditions (Fig. 4-3), suggesting that some endogenous LPA may exist or accumulate in the cellular microenvironment during the course of the experiments. Consistent with the greater basal migratory activity associated with loss of LPA_4, more *lpa4* KO MEFs than WT cells migrated towards EGF (Fig. 4-3). However, the net increase by EGF over unstimulated conditions was similar between *lpa4* KO and WT MEFs. Thus lack of LPA_4 specifically sensitized MEFs to LPA-induced chemotaxis. Furthermore, the enhancement of LPA-mediated cell migration in the absence of LPA_4 was consistent among multiple pairs of *lpa4* WT and KO MEFs and was observed in both primary MEFs and immortalized MEF lines (Fig. 4-4).

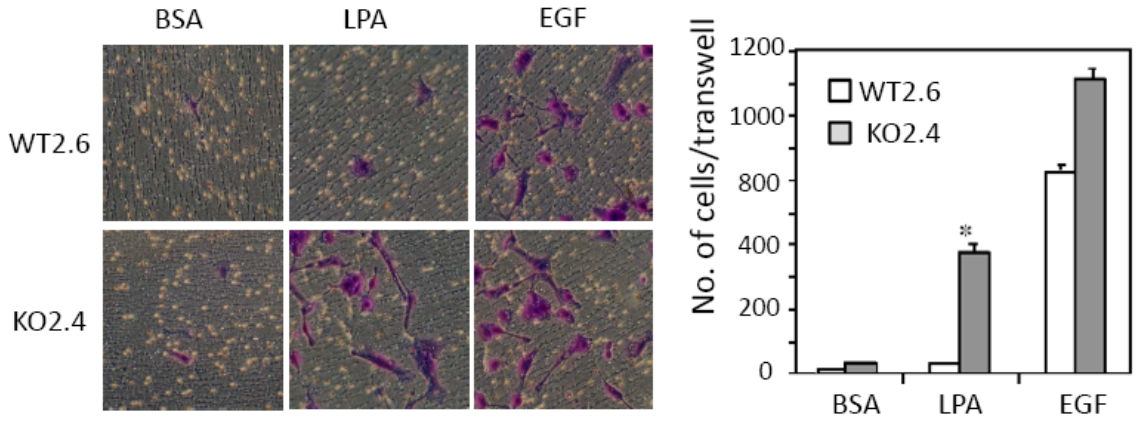


Figure 4-3. **Sensitization of LPA_4-deficient MEFs to migratory response to LPA.** The chemotactic response to LPA (5 μM) or EGF (25 ng/ml) in primary MEFs (WT2.6 and KO2.4) were analyzed using collagen-coated transwells. The cells (2.5×10^4 cells/0.5 ml) were loaded to the wells and allowed to migrate for 6 hours The migrated cells on the underside of transwells were stained with crystal violet and photographed under microscope (200 ×) (*left panel*). The LPA-induced chemotactic migration was quantified as detailed in Materials and Methods (*right panel*). The results were presented as average numbers of migrated cells per transwell ± SD of triplicates, representative of three independent experiments. *, $p = 0.000003$.

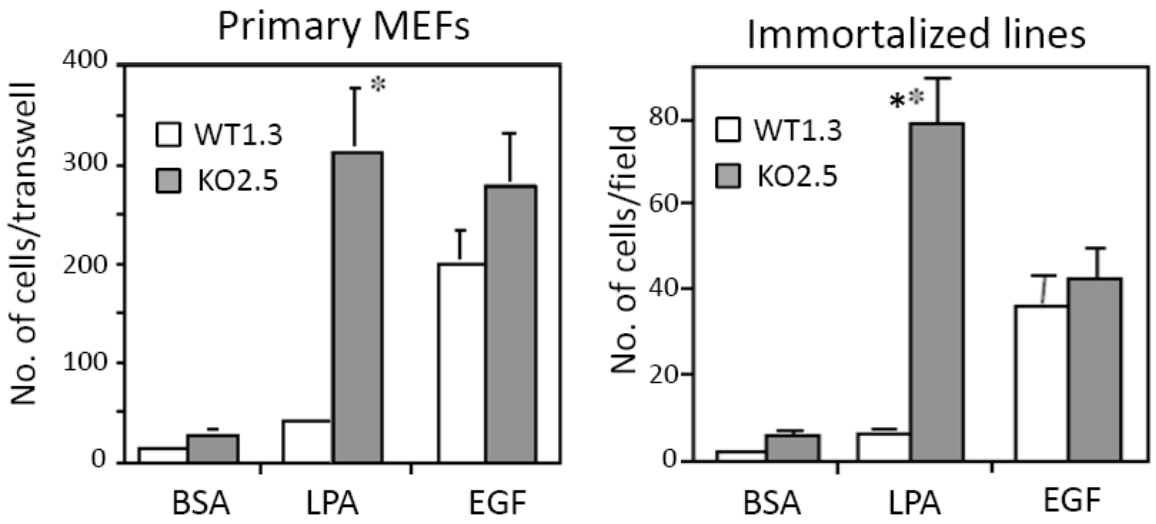


Figure 4-4. Sensitization of LPA_4-deficeint cells to LPA-induced cell migration. Chemotaxis responses to LPA and EGF were assessed in another independent pair of wild type (WT1.3) and KO (KO2.5) primary MEFs and immortalized lines. The results were presented as average numbers of migrated cells per transwell or per field (200 ×) ± SD of triplicates, representative of three independent experiments. *, p = 0.000007; **, p = 0.000091.

Potentiation of AKT and Rac activation by lpa$_4$ deletion

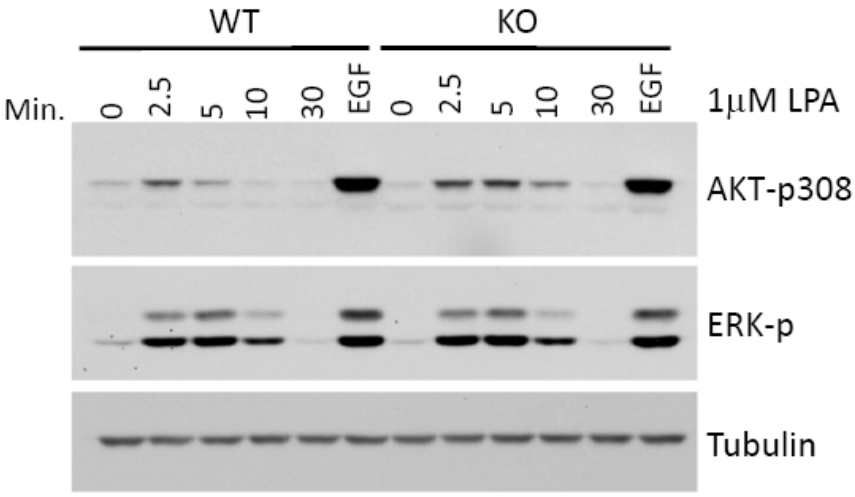
To understand functions of LPA_4 in cell signaling, we analyzed activation of AKT and ERK by LPA in WT and *lpa$_4$* KO MEFs. Although LPA-induced ERK phosphorylation was similar between WT and KO MEFs, LPA_4 deficiency resulted in enhancement of AKT phosphorylation at Thr-308 in response to LPA (Fig. 4-5A). The potentiating effect of LPA_4 deficiency on AKT activation was seen in multiple pairs of WT and KO MEFs (data not shown). Furthermore, the enhanced AKT activation in LPA_4-deficient cells was sensitive to LPA_1-selective antagonist Ki16425 (124), G_i inhibitor PTX, and the PI3K inhibitor LY294002 (Fig. 4-5B), indicating that the prominent AKT activation was mediated through the LPA_1-G_i-PI3K signaling route in LPA_4-deficient cells. Moreover, LPA-induced chemotactic migration of LPA_4-deficient cells was suppressed by Ki16425, PTX or LY294002 but only slightly reduced by an AKT inhibitor (AKTi II) (125), suggesting that an LPA_1-G_i-PI3K-dependent pathway mediates the robust migratory response to LPA in LPA_4-deficeint cells.

We next examined LPA-induced activation of Rac, a well-documented effecter of PI3K, and the $G_{12/13}$ downstream target Rho (26,60,74,91). Rac and Rho mediate cell migration in a coordinate fashion. Rac promotes lamellipodia protrusion and forward movement while RhoA regulates actomyosin-driven cytoskeleton contraction and detachment of the rear of migrating cells (126). LPA-induced Rac activation in fibroblasts is generally weak and has been best demonstrated in LPA receptor-overexpressing cells (91,127). As shown in Fig. 4-6A, Rac activation in response to LPA was hardly detectable in WT MEF line, which correlated with the weak migratory response to LPA in WT cells

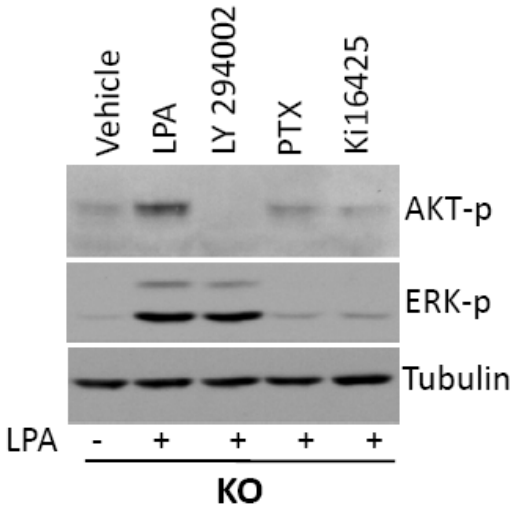
(Fig. 4-6B). In contrast, LPA evoked prominent increases in Rac-GTP levels in the *lpa*$_4$ KO MEF line (Fig. 4-6A).

In contrast to Rac activation, LPA-induced Rho activation was oppositely affected by LPA_4 deficiency. As shown in Fig. 4-6A, LPA induced immediate and sustained increases in Rho-GTP levels in both WT and *lpa*$_4$ KO MEF lines as measured by pull down with GST-Rhotekin Sepharose beads. However, the magnitude of Rho activation induced by LPA was significantly reduced in *lpa*$_4$ KO cells, suggesting that signaling through LPA_4 contributes to the overall Rho activation in LPA-stimulated cells. The result was consistent with the previous observation that in skin fibroblasts of LPA_1 and LPA_2 double knockouts, LPA remained capable of stimulating partial activation of Rho (62), which could be attributed to the input of the LPA_4 receptor.

(***A***)



(***B***)



(***C***)

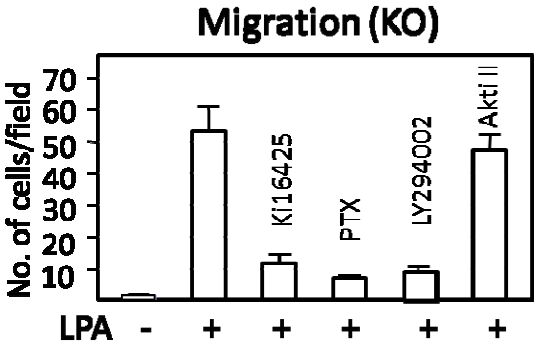


Figure 4-5. Sensitization of LPA_4-deficeint MEFs to LPA-induced AKT activation. The WT and KO MEF lines were starved in serum-free medium and stimulated with LPA (2.5 μM) for the indicated periods of time (***A***) or in the presence of the LPA_1 antagonist Ki16425 (10 μM), G_i inhibitor PTX (50 ng/ml) or PI3K inhibitor LY294002 (10 μM) (***B***). Phosphorylation of AKT at Thr308 and ERK at Thr202/Tyr204 of ERK1 and

Thr185/Tyr187 of ERK2 were analyzed by immunoblotting with AKT and ERK phospho-specific antibodies. LPA-induced chemotaxis in the KO MEF line was quantified in the absence or presence of the inhibitors described in (***C***) or AKT inhibitor (AKTi II) (10 μM).

(***A***) (***B***)

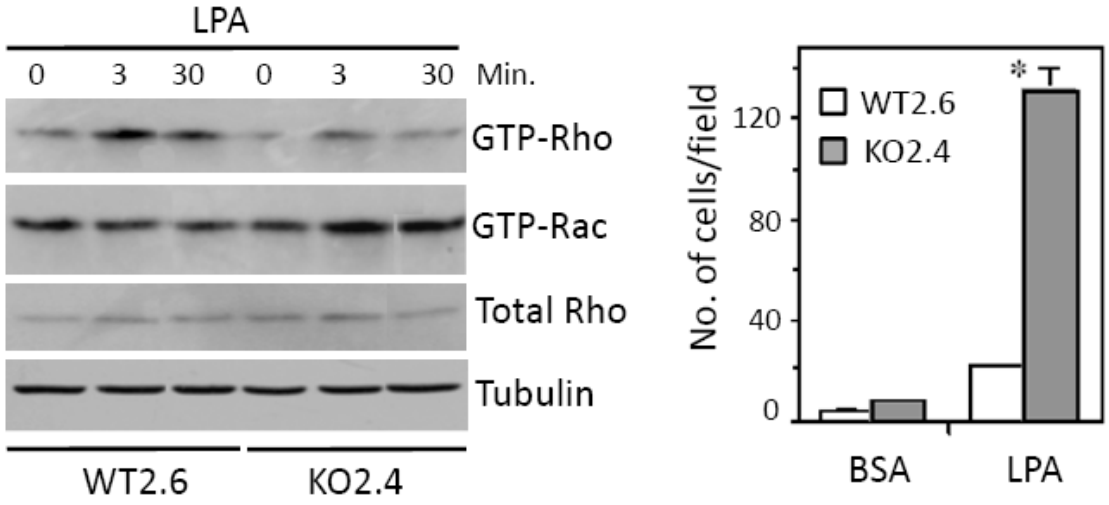


Figure 4-6. **Inhibition of Rho and potentiation of Rac activation by *lpa*$_4$ deletion: correlation with the effect on cell migration.** (***A***) The WT2.6 and KO2.4 cell lines were grown to subconfluence, starved in serum-free medium and stimulated with LPA (2.5 μM) for the indicated periods of time. The cells were lysed and analyzed for GTP-bound Rho or Rac with the GST pulldown assays as described in Materials and Methods. Immunoblotting analysis of the total Rho and tubulin was included as protein level controls. (***B***) Inhibition of Rho and potentiation of Rac activation correlated with the effect of LPA_4 deletion on cell migration. LPA-induced chemotaxis in the WT2.6 and KO2.4 lines was analyzed and quantified as described in Fig. 4-3. *, $p = 0.000042$.

Inhibition of cell motility by reconstitution of LPA_4

To ascertain whether the enhanced cell migration ability of *lpa4* KO cells in response to LPA was a direct result from the LPA_4 deficiency rather than nonspecific effects secondary to gene targeting or MEFs preparation, LPA_4 was restored in the KO MEFs using the lentivirus-mediated gene transduction. The re-expression of LPA_4 in KO MEFs was confirmed by western blotting analysis (Fig. 4-7). The LPA-induced migration in control virus-infected and LPA_4-reconstituted cells was compared. As shown in Fig. 4-8, LPA_4-reexpressing cells migrated in response to LPA less efficiently than the control virus-infected, LPA_4-negative cells, confirming that LPA_4 functions as a suppressor of LPA-dependent cell migration. Furthermore, the inhibition of LPA-dependent cell migration by re-expression of LPA_4 was accompanied by increased Rho but decreased Rac activation induced by LPA (Fig. 4-8).

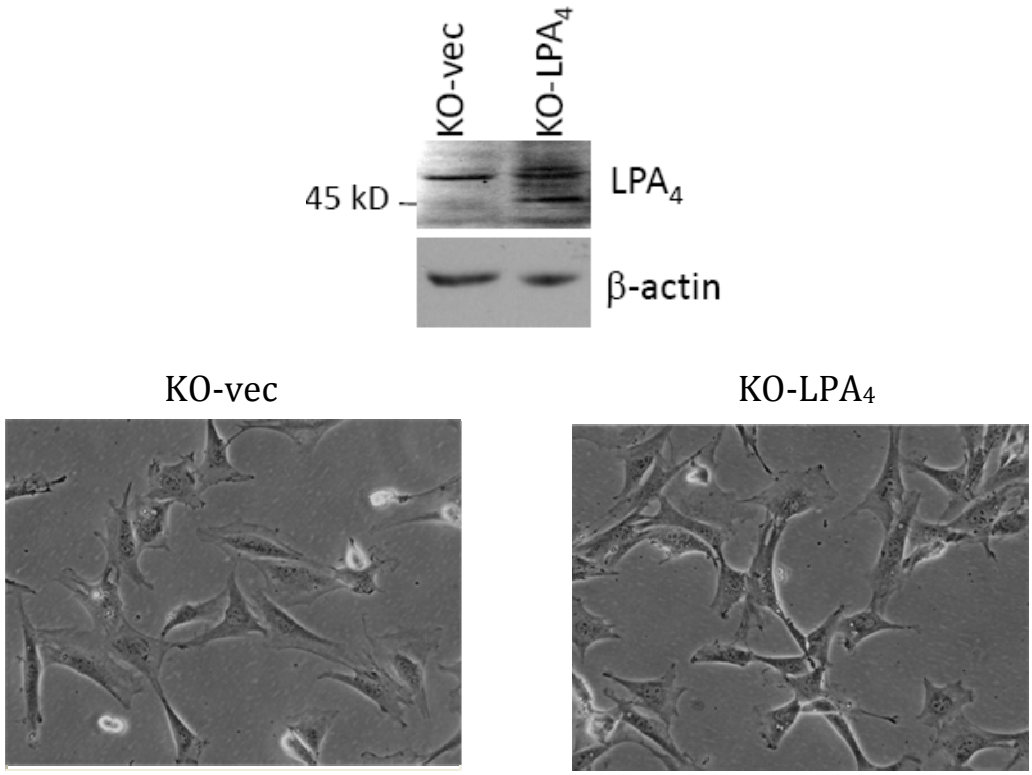


Figure 4-7. Reconstitution of LPA_4 in LPA_4-deficient MEFs. LPA_4 expression was reconstituted in KO2.5 MEF line by infection with pLenti-LPA_4 lentivirus. The expression of LPA_4 in transduced cells (KO2.5-LPA_4) but not in empty virus-infected cells (KO2.5-vec) was confirmed by immunoblotting with anti-LPA_4 antibody (*upper panel*). Phase contrast photographs under microscope of lentivirus transduced control vector or LPA_4 KO2.5 MEFs (*lower panel*).

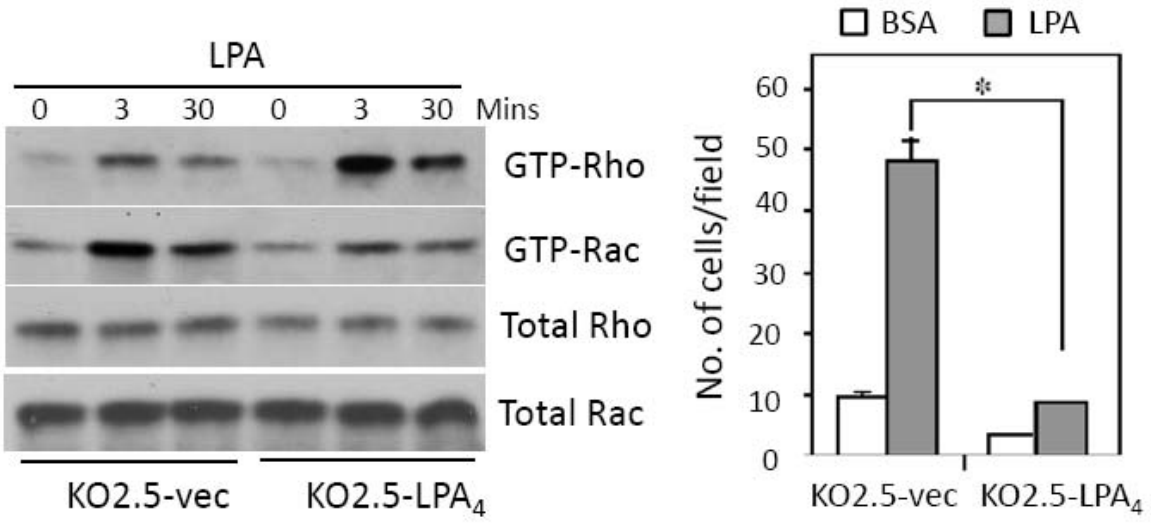
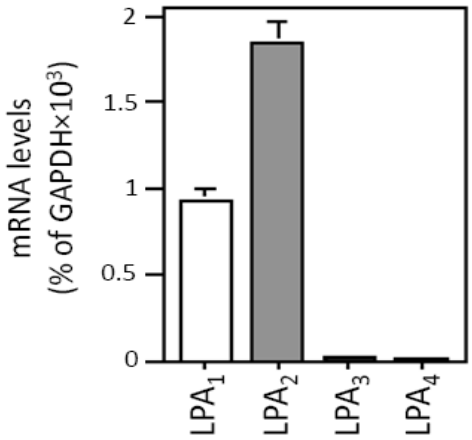


Figure 4-8. Desensitization of LPA_4-deficienct cells to LPA-induced migration by reconstitution of LPA_4. Expression of LPA_4 enhanced Rho but decreased Rac activation. LPA-induced Rho and Rac activation in the KO2.5-vec and KO2.5-LPA_4 cells was analyzed with the GST pull down assays as described in Fig. 4-6 (*left panel*). The chemotactic response to LPA in these cells was analyzed and quantified as described in Fig. 4-3 (*right panel*). *, $p = 0.000020$.

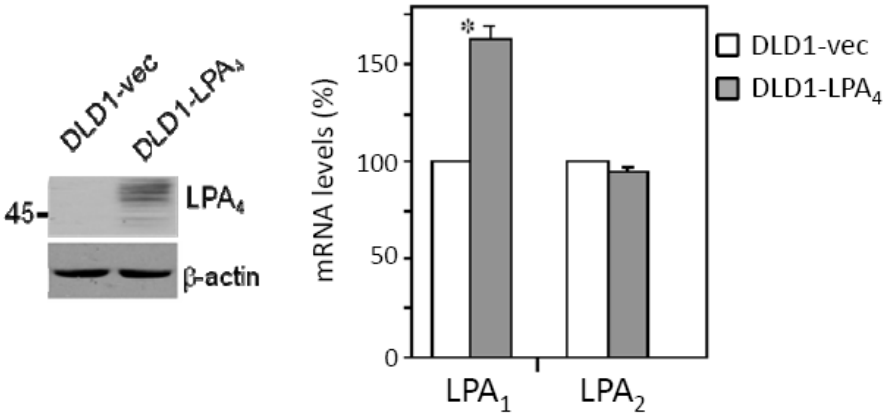
Suppression of LPA-induced cell migration and invasion by LPA_4 in colon cancer cells

To explore the physiological significance of the motility inhibitory effect of LPA_4, we assessed the role of LPA_4 in migration and invasion of human cancer cells. The DLD1 colon cancer cell line was previously shown to migrate efficiently in response to LPA (72). Sub-micromolar concentrations of LPA were sufficient to trigger significant migratory response to LPA in DLD1 cells. DLD1 cells expressed LPA_1 and LPA_2, but lacked LPA_3 or LPA_4 mRNA as analyzed by RT-qPCR (Fig. 4-9A). We introduced LPA_4 into DLD1 cells via lentivirus-mediated transduction (Fig. 4-9B). LPA-induced migration in DLD1 cells was dramatically inhibited by ectopic expression of LPA_4 (Fig. 4-9C). LPA was effective in stimulating invasion of DLD1 cells through the Matrigel Basement Membrane Matrix. The invasion induced by LPA was also significantly suppressed by expression of LPA_4 (Fig. 4-9C). The inhibitory effect of LPA_4 on migration and invasion of DLD1cells was not due to interference with endogenous LPA_1 or LPA_2 receptor expression. In fact, following introduction of LPA_4, LPA_2 mRNA expression was not altered and LPA_1 mRNA level was actually modestly increased as determined by RT-qPCR (Fig. 4-9B). Collectively, these results confirm a general role for LPA_4 in negative regulation of motility-stimulating activity of LPA in both normal and neoplastic cells.

(***A***)


mRNA levels
(% of GAPDH×10³)
2
1.5
1
0.5
0
LPA1
LPA2
LPA3
LPA4

(***B***)


DLD1-vec
DLD1-LPA4
45
LPA4
β-actin
mRNA levels (%)
150
100
50
0
*
LPA1
LPA2
DLD1-vec
DLD1-LPA4

(***C***)

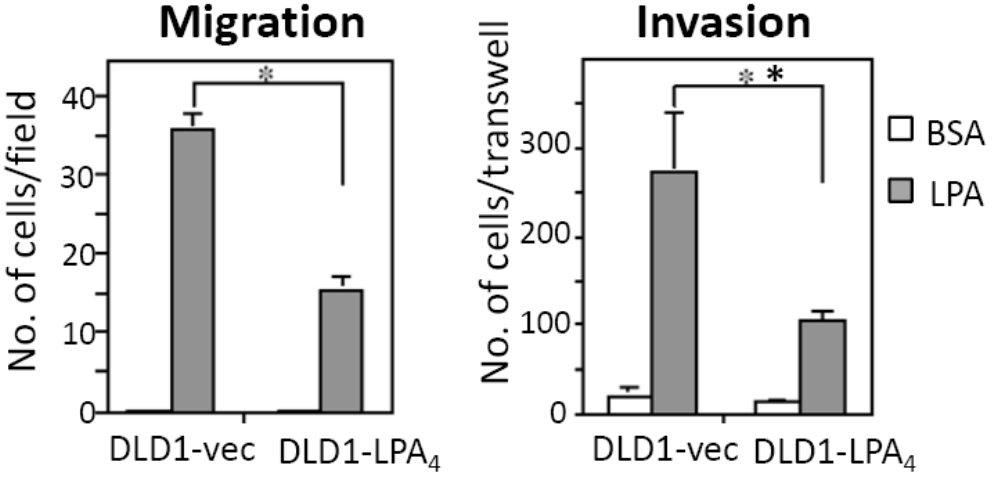

Migration
No. of cells/field
40
30
20
10
0
*
DLD1-vec
DLD1-LPA4
Invasion
No. of cells/transwell
300
200
100
0
* *
DLD1-vec
DLD1-LPA4
BSA
LPA

Figure 4-9. Inhibition of LPA-induced migration and invasion of the DLD1 colon cancer cells by LPA_4. (***A***) RT-qPCR analysis indicated expression of LPA_1 and LPA_2 but not LPA_3 or LPA_4 mRNA in DLD1 cells. The mRNA levels were presented as percentages relative to GAPDH. (***B***) Ectopic expression of LPA_4 did not decrease expression of motility-promoting Edg LPA receptors. Immunoblotting analysis confirmed the expression of transduced LPA_4 in DLD1-LPA_4 cells (*left panel*). Expression levels of endogenous LPA_1 and LPA_2 mRNAs in DLD1-vec and DLD1-LPA_4 cells were compared by RT-qPCR. The results were normalized on GAPDH and presented as relative percentages with the mRNA levels in the control DLD1-vec cells defined as 100%. (***C***) Expression of LPA_4 inhibited LPA-induced migration and invasion of DLD1 cells. The DLD1-vec and DLD1-LPA_4 cells (2.5×10^4 cells/transwell, 0.2 μM LPA) were allowed to migrate for 6 hr or to invade through Matrigel for 24 hr. The cell numbers, LPA concentrations and incubation times used in the experiments were determined empirically that yielded maximal or submaximal responses. The results of migration and invasion assays were presented as detailed in Fig. 4-3. *, $p = 0.00003$; **, $p = 0.0038$ (Student's *t* test).

Antagonism of LPA_1-dependent cell migration by LPA_4

Most mammalian cell types express more than one LPA receptor subtypes and respond to LPA, making it difficult to connect a cellular response to a specific LPA receptor. It is of interest to study the potential cooperation or antagonism among LPA receptor subtypes. The rat neuroblastoma cell line B103 offers an ideal model to examine the receptor crosstalk due to the absence of endogenous LPA receptors (107). Although it was recently shown that there was slight expression of LPA_4 mRNA in B103 cells, the cell line did not respond to LPA stimulation (60). Therefore, we infected B103 with retrovirus carrying LPA_4 or LPA_1, or with control retrovirus. The transduced, GFP-positive cells were isolated by FACS and expanded as stable lines expressing LPA_4 or LPA_1. In agreement with LPA_4 being a functional receptor for LPA, LPA_4-expressing B103 cells underwent neurite retraction and cell rounding upon exposure to LPA (see results in Fig. 2-9 of Chapter 2), similar to the response seen in LPA_1-expressing B103 cells. However, only LPA_1-expressing cells showed chemotactic response to LPA while the LPA_4-expressing cells and vector control cells did not (Fig. 2-10 of Chapter 2). The observation indicates that LPA_4 is not capable of driving cell migration in spite of its ability to promote Rho-dependent neurite retraction.

To determine whether LPA_4 counteracts the motility-stimulating action of LPA_1, we co-expressed LPA_1 and LPA_4 in B103 cells by sequential infection with pLenti-TOPO-LPA_1 lentivirus and pLZRS-EGFP-LPA_4 retrovirus. Co-expression of these LPA receptors was confirmed by western blotting (Fig. 4-10). Interestingly, LPA_4 expression indeed inhibited LPA_1-mediated migration of B103 cells induced by LPA. The LPA_1-dependent

migration was inhibited by co-expressed LPA_4 by 40%, not as strongly as seen in LPA_4-reexpressing MEFs or LPA_4-transduced DLD1 cells probably because the co-expressed LPA_1 receptor was abundant in the recipient cells (Fig. 4-11). High levels of LPA_1 could provide a strong motility-promoting drive upon LPA stimulation. In addition, B103 cells expressing LPA_1 was capable of invading through Matrigel in response to LPA. This effect was also significantly attenuated in the cells co-expressing LPA_4 (Fig. 4-11).

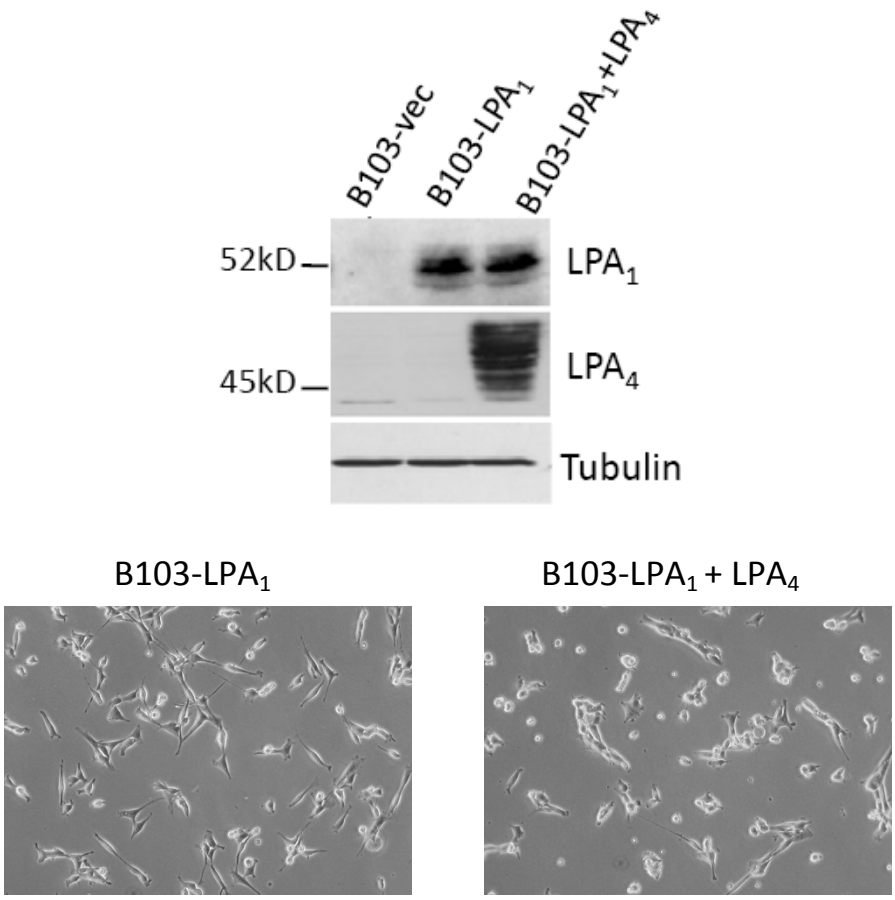


Figure 4-10. Stable co-expression of LPA_1 and LPA_4 results in distinct morphology. B103 cells were stably transduced with vector (B103-vec), LPA_1 (B103-LPA_1) or both LPA_1 and LPA_4 (B103-LPA_1 + LPA_4). Expression of each receptor was confirmed by Western blot analysis (*upper panel*). Phase-contrast images of B103-LPA_1 and B103-LPA_1+LPA_4. Note that B103 cells co-expressing LPA_1 and LPA_4 appeared to adhere to each other forming aggregation compared to expression of LPA_1 alone (*lower panel*).

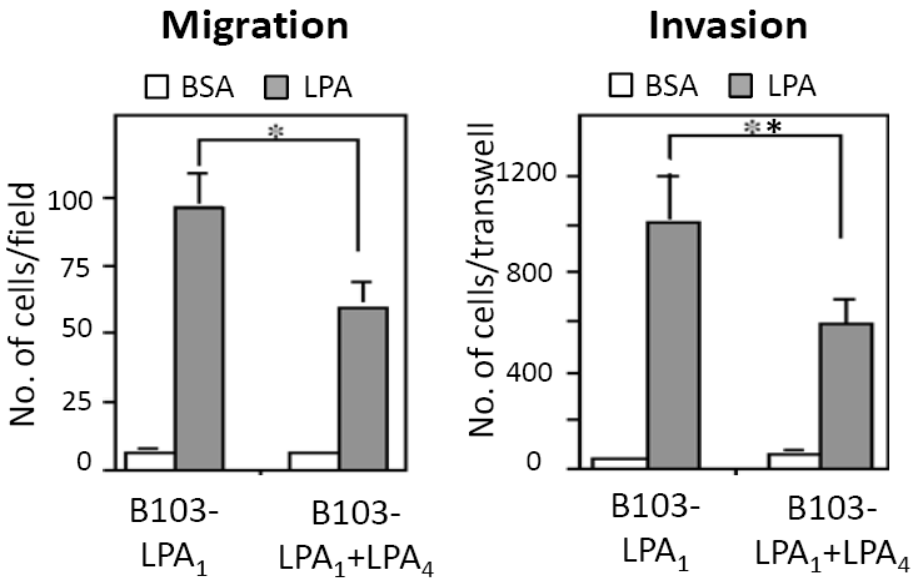


Figure 4-11. Antagonism of LPA_1-dependent cell migration by LPA_4. LPA_4 inhibited LPA_1-mediated migration and invasion when co-expressed with LPA_1. B103 cells were transduced with vector (B103-vec), LPA_1 (B103-LPA_1) or both LPA_1 and LPA_4 (B103-LPA_1 + LPA_4). The chemotactic migration induced by LPA in these cells was analyzed under the same conditions as in Fig. 2-10 (*left panel*). The *in vitro* invasion assays were performed with LPA (1 μM) and 1×10^4 cells/transwell coated with Matrigel as described in Fig. 4-9 (*right panel*).

DISCUSSION

Six GPCRs have been identified as cognate LPA receptors to mediate a multitude of cellular responses to LPA (5,52-56). It remains elusive why there are multiple receptor subtypes for the small lipid mediator. It is even more intriguing that these LPA receptors fall into structurally distant families sharing little amino acid identities. LPA_{1-3} and the receptors for the closely related S1P ($S1P_{1-5}$) belong to the Edg family (26,58). The LPA_1 receptor is most widely distributed and is involved in initiation of neuropathic pain and promotion of pulmonary and renal fibrosis (64-66). LPA_3, a receptor subtype expressed in the uterus, seems to be important for embryo implantation and spacing (83). LPA_2 and LPA_3 receptors are overexpressed in a variety of human malignancies including cancers of the colon, ovary, breast and thyroid (71,73,82,128). The LPA_4 receptor is not only structurally distant from the LPA_{1-3} receptors of the Edg family, but also couple to different categories of G proteins. It is linked to G_q, $G_{12/13}$ and probably G_s, but not G_i whereas LPA_{1-3} receptors all couple to G_i (26). The differences in receptor structure and G protein partnership between the Edg LPA receptors and the LPA_4 receptors prompted us to study biological functions of LPA_4 by developing knockout mice. While the interim characterization has not revealed apparent abnormalities in LPA_4-deficient mice, the truly LPA_4-negative MEFs isolated from LPA_4-deficient embryos have allowed us to evaluate the role of LPA_4 in LPA signal transduction and migratory response to LPA. The results presented in the current work clearly demonstrate that knockout of *lpa4* sensitizes the cells to LPA-induced migration. In WT cells, the presence of LPA_4 may thus serve as a barrier preventing overreaction to LPA. The specific role of LPA_4 in limiting LPA-induced cell

motility was confirmed by LPA_4 reconstitution experiment showing reexpression of LPA_4 desensitizes KO MEFs to LPA-induced migration. Moreover, heterologous expression of LPA_4 in rat neuroblastoma and human colon cancer cell lines suppressed LPA-triggered cell migration and invasion, antagonizing the motility-promoting effect of the LPA_1 receptor. It will be of interest to examine in the future whether LPA_4 expression was deregulated in human tumors contributing to the invasive and metastatic potential of cancer cells.

The discovery of negative regulation of LPA-dependent migration by LPA_4 provides novel insights into the crosstalk among the multiple LPA receptors that are frequently co-expressed in mammalian cells. We have recently described that LPA induced cyclooxygenase-2 expression in ovarian cancer cells through a mechanism requiring LPA_1, LPA_2, and LPA_5 receptors (24). Integration of signals from multiple LPA receptors may be necessary for the optimal activation of LPA-mediated responses. There are many other examples of functional complementation or redundancy among LPA receptors (19,24,107,129). However, the functional antagonism between LPA receptors has rarely been demonstrated (107). The opposing effects of LPA_1 and LPA_4 receptors on LPA-induced cell migration and invasion described in the current work represent such an example of functional inhibition among LPA receptors. This inhibitory crosstalk is likely critical to ensure physiologically appropriate responses to LPA. It is yet to be determined whether LPA_4 also negatively regulate some other biological functions of LPA. In B103 cells, however, LPA_4 mimics LPA_1 in promoting neurite retraction and cell rounding,

suggesting that LPA_4 is indeed a functional LPA receptor that mediates certain cellular effects of LPA while inhibiting others, depending on signaling pathways involved.

Although the molecular mechanism for LPA_4 downregulation of cell motility remains to be fully elucidated, our results provide some clues to the potential players in the process. As discussed above, the cell motility is tightly controlled by activities of Rho and Rac in a coordinate fashion (126).The balance of Rho and Rac activities are critical determinants of cell movement (126). LPA_4 contributes to the total Rho activation in MEFs likely through $G_{12/13}$ (59.60). Our results demonstrate that loss of LPA_4 decreased LPA-stimulated Rho activation as anticipated but simultaneously enhanced LPA-induced Rac activation. The observation suggests that LPA_4 may exert its inhibitory effect on cell migration through increasing the relative ratios of active Rho versus active Rac in LPA-treated cells. LPA_4 seems to interfere with activation of Rac by inhibiting PI3K. This is supported by the fact that LPA_4 expression attenuates other PI3K effectors such as AKT. In addition to this possibility, Rac activation could be inhibited by excessive Rho activity in WT cells as has been proposed in other cell systems (95,130,131). It is also possible that LPA_4 directly desensitizes the LPA_1 receptor that is known to stimulate Rac activation through G_i (91)**.** The role of LPA_4 in negative control of cell motility is reminiscent of $S1P_2$, one of S1P receptors, that has been shown to inhibit cell migration (95,131,132). Similar to LPA_4, $S1P_2$ is coupled to $G_{12/13}$ and G_q, but not G_i (58). Several studies suggest the negative effect of $S1P_2$ on cell motility is attributed to $G_{12/13}$-mediated Rho activation (95,131,132). Activation of Rho in the absence of appropriate Rac input seems to be sufficient to confer inhibition of cell motility (95,130,131).

It is not surprising that the LPA_4-deficient mice do not show obvious phenotypic abnormalities at least at early ages. LPA_1 and LPA_2 knockouts are also dispensable from normal development and physiology (63,79). However, these knockout animals have proved to be valued models to link LPA signaling to pathophysiological processes (64-66). The backup and/or redundant receptor subtypes of LPA may suffice to compensate for the loss of individual LPA receptors *in vivo*. Alternatively, LPA, as one of phospholipids present in the circulation and tissues, may not be the only or rate-limiting mediator physiologically required *in vivo*. Instead, LPA signaling may be more critical in pathophysiological settings when levels of the lipid mediator are locally and temporally altered. Recent studies of LPA_1-deficeint mice demonstrated involvement of LPA_1 in abnormal wound healing and fibrosis formation (64,65) supports a major role for this LPA receptor subtype in chemotactic recruitment of fibroblasts to the site of wound. In light of the opposing effects of LPA_4 and LPA_1 on migratory response of various cell types to LPA, it is interesting to study roles of LPA_4 in wound healing and other pathophysiological conditions using LPA_4-deficient mice developed in the current study.

CHAPTER 5: GENERAL DISCUSSION AND FUTURE DIRECTIONS

Major Findings and Achievements

The present study aimed to gain better understanding of LPA_4, one of the newly identified LPA receptors. The three new LPA receptors (LPA_{4-6}) are structurally diverse from the well defined Edg LPA receptors. Little is known about their expression and roles in LPA signal transduction, health and diseases. Comprehensive knowledge of LPA_4 will provide clues to the physiological roles of this new subclass of LPA receptors. Our results confirmed the identity of LPA_4 as a functional LPA receptor which mediates a subset of responses to LPA. Functional studies of LPA_4 demonstrate that LPA_4 couples primarily to $G_{12/13}$, which activates the Rho-ROCK pathway and actomyocin-driven cytoskeleton remodeling. Different from the Edg LPA receptors, LPA_4 fails to activate G_i-mediated ERK and PI3K pathways and the subsequent migratory response to LPA. To delineate physiological functions of LPA_4, we have developed *lpa4* knockout mice. Interim analysis of *lpa4* knockout mice showed that LPA_4 is not required for embryonic development or fertility. The LPA_4-deficient mice are physiologically normal and are grossly indistinguishable from their wild type littermates. However, these animals provide a useful cell model to study the effect of LPA_4 deficiency on LPA signal transduction and responses to LPA. Our analysis demonstrated a novel function of LPA_4 in suppressor of cell motility in contrast to the Edg LPA receptors that all positively regulate cell migration.

Deletion of *lpa4* dramatically sensitizes cells to LPA-induced cell migration. On the other hand, overexpression of LPA_4 inhibits LPA-mediated cell migration and tumor cell invasion. When coexpressed with the motility-stimulating LPA_1 receptor, LPA_4 attenuates the LPA_1-driven cell migration and invasion, highlighting functional antagonism between the two subtypes of LPA receptors. These results constitute the first evidence for the existence of negative LPA receptors. The crosstalk among LPA receptors may be critical to ensure physiologically appropriate responses to LPA. Mechanistic studies of *lpa4* knockout and "knock-in" MEFs further demonstrated that LPA_4 inhibits activation of PI3K-dependent activation of AKT and Rac, an effect that could suppress cell motility.

Potential Mechanism for Negative Regulation of PI3K by LPA_4

LPA_4 does not couple to G_i and G_i-dependent activation of PI3K. However, it remains to be determined how expression of LPA_4 negatively impinges on LPA-induced activation of PI3K and the downstream effectors AKT and Rac. One possibility is that LPA_4-triggered activation of the G_q-PLCβ competes with G_i-PI3K for their common substrate PIP2. Since LPA_4 couples to G_q only, loss of LPA_4 should allow PIP2 accumulation for PI3K to convert to PIP3, which favors activation of the downstream targets AKT and Rac. This possibility could be evaluated by supplying excessive exogenous PIP2 or inhibition of PLCβ with specific pharmacological inhibitors. Another possible mechanism for LPA_4 negative regulation of PI3K is desensitization of the Edg LPA receptors through physical interactions such as hetero-dimerization or other types of complexion. It has been reported that LPA receptors have tendency to form homo- and

hetero-dimers with each other, with S1P receptors and even with certain unrelated GPCRs (133). So it is likely that LPA_4 may form hetero-dimers with the PI3K-stimulating LPA_{1-3} receptors. As such, the presence of LPA_4 will interfere with LPA-dependent activation of AKT and Rac. We will examine the possibility of physical interactions between LPA_4 and the Edg LPA receptors by co-immunoprecipitaion and the LacZ complementation assays (133).

Role of LPA_4 in Prevention of Cancer Metastasis

There is substantial evidence that LPA plays a role in tumor cell invasion and metastasis. As stated before, the LPA-producing enzyme ATX was originally identified as a tumor cell motility-stimulating factor (39). Overexpression of ATX in Ras-transformed 3T3 cells enhances metastasis of xenografts in nude mice (134). In further support of a role of LPA signaling in cancer metastasis, expression of LPA_1, the major chemoattractant receptor for LPA, enhances bone metastasis of breast cancer cell lines in nude mice (135). Conversely, downregulation of LPA_1 expression or blockade of LPA_1 with a selective antagonist (Ki16425) inhibited metastasis of these cells to the bone (135,136). If LPA_4 is a suppressor of motility of both normal and neoplastic cells, LPA_4 could be downregulated in human cancers and their metastasis. We are currently testing this possibility by profiling LPA_4 expression in various human cancers and their normal tissue counterparts. At present, we know that LPA_4 expression is reduced at least in ovarian cancer cell lines compared to normal ovarian epithelial cells.

LPA_4 Knockout: Lack of Phenotypes or Lack of Thorough Analysis

In spite of lack of apparent phenotypes, we have used the cells from the *lpa₄* KO mice to uncover a previously unrecognized function of LPA_4. It is unknown why loss of LPA_4 and its inhibitory effect on cell motility does not culminate in any major physiological defects in mice at least at early ages. Similarly, *lpa₁* and *lpa₂* knockouts are also dispensable from normal development and physiology despite the significant deficit in cell signaling associated with deletion of *lpa₁* or *lpa₂* (63,79). However, these *lpa₁* knockout animals have proved to be valued models to link LPA signaling to many pathophysiological processes (64-66). The phenotypes of LPA receptor-deficient mice are compatible with involvement of LPA signaling in pathophysiological circumstances. Hence we will further explore the phenotypes of LPA_4-deficient mice under stress as has been done with *lpa₁* knockout mice (64,65).

Based upon the crucial roles of the PI3K- AKT pathway in oncogenesis, deletion of *lpa₄* and its inhibitory effect on PI3K may increase the susceptibility of the LPA_4-deficient mice to spontaneous or carcinogen-induced tumorigenesis. We have indeed observed development of various spontaneous tumors in *lpa₄* KO and heterozygous mice, but not in their wild type littermates. Further analysis involving more animals and chemical carcinogens will help provide definitive answer to whether loss of LPA_4 leads to increased susceptibility to tumorigenesis.

LPA_4 as a Therapeutic Target

As addressed earlier, LPA_4 likely functions as a negative regulator of migration, invasion and metastasis of tumor cells. There is also preliminary evidence that LPA_4 may act as a tumor suppressor. As a cell surface GPCR, LPA_4 is a highly druggable target. It is well known that more than half of medicines in the current use target GPCRs. It is possible to identify LPA_4-specific agonists that do not cross react with the structurally distant Edg LPA receptors. A sensitive and robust GPCR activation assay such as the recently developed Tango™ assay could be used to screen LPA analogs (137). The LPA_4-selective agonists may be appealing chemoprevention and anti-neoplastic agents if the tumor-suppressive function of LPA_4 is confirmed.

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PUBLICATIONS

Lee Z, Swaby RF, Liang Y, Yu S, Liu S, Lu KH, Bast RC Jr, Mills GB, and Fang X. (2006) Lysophosphatidic acid is a major regulator of growth-regulated oncogene alpha in ovarian cancer. Cancer Res. 66, 2740-2748

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Lee Z, Cheng C, Zhang H, Subler MA, Wu J, Windle JJ, Chen C-K, and Fang X. Role of LPA_c/GPR23/p2y9 in negative regulation of cell motility. Submitted, Molecular Biology of the Cell 2008

Song Y, Jinhua Wu, **Lee Z**, Oyesanya R, Mukherjee A, and Fang X. Lysophosphatidic acid induces expression of VEGF via c-Myc and Sp1 transcription factors: implications for tumor angiogenesis (manuscript in preparation).

ABSTRACTS AND PRESENTATIONS

Lee Z, Liang Y, Yu S, Liu S, Bast RC Jr, Mills GB, and Fang X. Identification of lysophosphatidic acid as a major regulator of growth-regulated oncogene α (GROα) in ovarian cancer. Poster presentation at the 40th Annual South Eastern Regional Lipid Conference (SERLC), 2005

Lee Z, Cheng C, Cheng J, Lynch K, and Fang X. LPA_4/GPR23, a Novel Receptor for Lysophosphatidic Acid, is involved in Cellular Growth and Transformation. Poster presentation at Integrative Cellular and Molecular Signaling Research Symposium, 2005

Lee Z, Swaby RF, Liang Y, Yu S, Liu S, Lu KH, Bast RC Jr, Mills GB, and Fang X. Identification of Lysophosphatidic Acid as a Major Regulator of Growth-regulated Oncogene α (GRQα) in Ovarian Cancer. Oral presentation at the 33rd Annual John D. Forbes Graduate Student Honors Colloquium, 2005

Lee Z, Cheng C, Cheng J, Lynch K, and Fang X. Role of LPA_4/GPR23, a Novel Receptor for Lysophosphatidic Acid, in Cell Growth, Survival and Transformation. Poster presentation at Daniel T. Watts Research Symposium, 2006

Lee Z, Cheng C, Cheng J, Lynch K, and Fang X. Role of GPR23/LPA_4, a Novel Receptor for Lysophosphatidic Acid, in Cell Growth and Transformation. Presentation at the 34th Annual John D. Forbes Graduate Student Honors Colloquium, 2006

Oyesanya R, Chen J, **Lee Z**, Song Y, Kordula T, and Fang XJ. Regulation of Cox-2 expression by LPA involves a permissive signal from receptor tyrosine kinase and histone deacetylation. The 41th Annual Southeast Regional Lipid Conference. Cashiers, NC, Nov. 1-3, 2006

Song Y, Wilkins P, Hu W, Murthy KS, Chen J, **Lee Z**, Oyesanya R, Wu J, Barbour SE, and Fang X. Inhibition of calcium-independent phospholipase A2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells. The 97th AACR Annual Meeting, Washington DC, April 1-5, 2006

AWARDS

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